USE OF THE GREEN FLUORESCENT PROTEIN AS A SCREENABLE MARKER FOR PLANT TRANSFORMATION

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Inventor:	GORDON-KAMM WILLIAM (US); PIERCE DORTOTHY A (US); BIDNEY DENNIS (US); MILLER MIKE (US); ROSS MARGIT (US); WANG LIJUAN (US); BOWEN BENJAMIN (US); SANDAHL GARY (US); SCELONGE CHRISTOPHER (US)	EP0904371 (A3) EP0904371 (A3) EP0904371 (A2) more >>
Applicant:	GORDON KAMM WILLIAM (US); PIERCE DORTOTHY A (US); PIONEER HI BRED INT (US); BIDNEY DENNIS (US); MILLER MIKE (US); ROSS MARGIT (US); WANG LIJUAN (US); BOWEN BENJAMIN (US); SANDAHL GARY (US); SCELONGE CHRISTOPHER (US)	Cited documents: WO9627675 WO9403619 WO9201370 WO9301283 WO9417176 more >>

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Abstract of **WO9741228**

A method for the production of transgenic plants is provided in which a vector carrying a gene encoding the green fluorescent protein is introduced into cells, the cells are screened for the protein and transformed cells are selected and regenerated. The cellular toxicity of the green fluorescent protein is circumvented by regulating expression of the gene encoding the protein or directing the protein to a subcellular compartment where it is not toxic to the cell. DNA constructs are provided for cell transformation in which the expression of a gene encoding the green fluorescent protein is placed under the control of an inducible promoter. In addition, DNA constructs are provided in which a nucleotide sequence encoding the green fluorescent protein is operably linked to a signal sequence which directs the expressed protein to a subcellular compartment where the protein is not toxic to the cell. Oxidative stress to plant cells transformed with GFP also can be ameliorated by transforming cells with an expression vector comprising genes encoding GFP and an oxygen scavenger enzyme such as superoxide dismutase. The toxicity of GFP in transformed plants can be eliminated by excising the screenable marker gene following detection of transformed cells or sectors. The FLP/FRT system is used in conjunction with GFP as a visible marker for transformation and FRT excision. A nucleotide sequence optimized for expression of the green fluorescent protein in plants is also provided.

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Description of **WO9741228**

USE OF THE GREEN FLUORESCENT PROTEIN AS A SCREENABLE
MARKER FOR PLANT TRANSFORX;TION
BACKGROUND OF THEINvEfiTON
1. Field of the Invention
The present invention relates to a method of plant transformation in which a DNA construct carrying a
gene encoding the green fluorescent protein (GFP) is introduced into plant cells which are then screened
for the presence of the protein and transformed cells are regenerated into transgenic plants. In particular,
the present invention provides methods for circumventing the cellular toxicity of the GFP by regulating
expression of the gene encoding the protein or directing the protein to a subcellular compartment where
it is not toxic to the cell. The present invention provides DNA constructs for cell transformation in which
expression of a gene encoding the GFP is placed under the control of an inducible, constitutive or tissue-
specific promoter. In addition, DNA constructs are provided in which a nucleotide sequence encoding the
GFP is operably linked to a signal or targeting sequence which directs the expressed protein to a
subcellular compartment where the protein is not toxic to the cell. Moreover, the present invention
provides a nucleotide sequence encoding GFP that is optimized for expression c @ the GFP gene in
plants and to GFP-encoding nucleotide sequences that code for light-shifted versions of GFP. The
present invention also provides a method for selecting plant cells transformed with a gene encoding a
screenable marker flanked on the 5-prime and 3-prime ends with a recombinase-specific target
sequence, and introducing a gene encoding a site specific recombinase into the transformed plant cells
and selecting transformed plant cells that no longer express the screenable marker. In addition, the
present invention provides a method of reducing GFP toxicity by transforming plant cells with a gene

encoding the GFP together with a gene encoding an oxygen scavenger such as superoxidase dismutase.

2. Background

Expression vectors include at least one genetic marker that allows transformed cells to be either recovered by negative selection, i.e. inhibiting. growth of cells that do not contain the selectable marker gene, or by screening for product encoded by the genetic marker. Many of the commonly used selectable marker genes for plant transformation were isolated from bacteria and code for enzymes that metabolically detoxify a selective chemical agent which may be an antibiotic or a herbicide. Other selectable marker genes encode an altered target which is insensitive to the inhibitor.

The most commonly used selectable marker gene for plant transformation is the neomycin phosphotransferase
II(nptII) gene, isolated from Tn5, which when placed under the control of plant regulatory signals confers resistance to kanamycin. Fraley et al., Proc. Natl.

Acad. Sci. U.S.A., 80: 4803 (1983). Another commonly used selectable marker gene is the hygromycin phosphotransferase gene which confers resistance to the antibiotic hygromycin. Vanden Elzen et al., Plant Mol.

Biol., 5: 299 (1985).

Additional selectable marker genes of bacterial origin that confer resistance to antibiotics include gentamycin acetyl transferase, streptomycin phosphotransferase, aminoglycoside-3'-adenyl transferase, the bleomycin resistance determinant. Hayford et al.,
Plant Physiol. 86: 1216 (1988), Jones et al., Mol. Gen.

Genet., 210: 86 (1987), Svab et al., Plant Mol. Biol. 14: 197 (1990), Hille et al., Plant Mol. Biol. 7: 171 (1986).

Other selectable marker genes confer resistance to herbicides such as glyphosate, glufosinate or broxynil.

Comai et al., Nature 317: 741-744 (1985), Gordon-Kamm et al., Plant Cell 2: 603-618 (1990) and Stalker et al.,
Science 242: 419-423(1988)
Other selectable marker genes for plant transformation are not of bacterial origin. These genes include, for example, mouse dihydrofolate reductase, plant5- enolpyruvylshikimate -3-phosphate synthase and plant acetolactate synthase. Eichholtz et al., Somatic
Cell Mol. Genet. 13: 67 (1987), Shah et al., Science233: 478(1986), Charest et al., Plant Cell Rep. 8: 643(1990)
Although many of these markers have been used for selecting transformed plant tissue, these selection systems involving toxic chemical agents can have disadvantages or limitations. One disadvantage is that it may be difficult to recover normal, viable transformed plants directly from chemical selection. Everett et al.,Bio/TechnologyS: 1201-1204(1987). Another disadvantage is that not all selectable marker systems work for all tissues, in all plant species, due in part to differences in sensitivity of a particular tissue or plant species to the selective agent. The success of any given marker for transformation of a given plant species is not easily predicted. Moreover, potential regulatory issues surrounding the use of antibiotic resistance genes and the use of herbicide resistance genes for plant species capable of outcrossing with weedy species are additional disadvantages of these markers.

Another class of marker genes for plant transformation require screening of presumptively transformed plant cells rather than direct genetic selection of transformed cells for resistance to a toxic substance such as an antibiotic. These genes are particularly useful to quantify or visualize the spatial pattern of expression of a gene in specific tissues and are frequently referred to as reporter genes because they can be fused to a gene or gene regulatory sequence for the investigation of gene expression. Commonly used genes for screening presumptively transformed cells includess-glucuronidase (GUS), P-galactosidase, luciferase, and chloramphenicol acetyltransferase.

Jefferson, R.A., Plant Mol. Biol. Rep. 5: 387 (1987).,
Teeri et al., EMBO J. 8: 343 (1989), Koncz et al., Proc.

Natl. Acad. Sci. U.S.A. 84: 131(1987), De Block et al.,
EMBO J. 3: 1681 (1984). Another approach to the identification of relatively rare transformation events has been use of a gene that encodes a dominant constitutive regulator of the Zea mays anthocyanin pigmentation pathway. Ludwig et al., Science 247: 449(1990)

Although chemical selection of plant cells transformed with selectable marker genes has been successful with plant species and varieties that are easily cultured in vitro, the choice of selectable marker systems that have been shown to be successful for cereals and many other agronomically important plant species is very limited. In general, plant species that tend toward organogenesis and/or shoot propagation have been difficult to transform by means of chemical selection.

The success rate with these plant species continues to improve, however, as evidenced by recent advances in Type
I selection of maize inbreds and small grain cereals such as barley. Koziel et al., Bio/Technologyii: 194-200 (1993) and Mendel et al. In: Transformation of Plants and
Soil Microorganisms, Wang et al. eds., Cambridge Press(1995).

Likewise, there has been little success in using visual screening methods for primary identification of transformed cells. The GUS gene was used to investigate germline transmission. McCabe et al., Plant Physiol., 87(3): 671 (1988) and McCabe et al., Plant Cell Tissue
Organ Cult. 33(3): 227 (1993). Histochemical staining for GUS activity was used to locate transgenic sectors in cotton and soybean transformants that ultimately produced transgenic seeds. Since histochemical analysis for GUS activity requires destruction of portions of the presumptively transformed plant tissue, this method is labor intensive and impractical for routine production of transgenic plants. This method is particularly unsuitable for plant species such as maize and other cereals in which transformants are recovered, even under optimum conditions, at low frequency. Recovery of transformed progeny was reported once in barley using GUS expression as a screening tool, but the method was found to be very labor-intensive. Ritala et al., Plant Mol.

Biol. 24: 317-325 (1994). There have been no reports at all of success with GUS or other screenable markers with maize.

More recently, in vivo methods for visualizing GUS activity that do not require destruction of plant tissue have been made available. Molecular Probes Publication 2908, Imagene Green", p. 1-4 (1993) and Naleway et al.,
J. Cell Biol.115: 151a (1991). However, these in vivo methods for visualizing GUS activity have not proven useful for recovery of transformed cells because of low sensitivity and high fluorescent backgrounds.

Despite the fact that luciferase genes have been available for many years, this strategy for visualizing transformed cells has also not been successfully adapted for routine recovery of plant transformants. The luciferase-based screening methods are limited by the fact that most of these systems require the presence of luciferin, a compatible luciferase and an exogenously supplied cofactor. In the absence of the substrate, enzyme or cofactor, the system does not bioluminesce.

Cells transformed with a luciferase gene must have cell walls and plasma membranes that are permeable, or rendered permeable, to a compatible luciferin in order to detect bioluminescence. For example, tobacco plants regenerated from cells transformed with a firefly luciferase gene and exposed to a liquid medium containing firefly luciferin exhibited bioluminescence primarily along their major veins. Ow et al., Science 234: 856 (1986). Accordingly, a screening method has not been successfully developed for routine plant transformation that does not involve chemical selection or assays, often labor-intensive, that require the sacrifice or destruction of tissue samples for analysis.

A gene encoding GFP has been utilized as a marker for gene expression in prokaryotic and eukaryotic cells.

Chalfie et al., Science 263: 802 (1994). Many cnidarians utilize GFPs as energy-transfer acceptors in bioluminescence. A gene encoding GFP isolated from a cnidarian and expressed in a heterologous prokaryotic or eukaryotic host produces a protein capable of fluorescence. A cDNA encoding the Aequorea victoria GFP produced a fluorescent product when expressed in
Escherichia coli or Caenorhabditis elegans cells. Green fluorescence was detected in transformed cells upon illumination with a long-wave ultraviolet (UV) source without having to supply substrates or cofactors. In addition, fluorescence was stable for at least 10 min when illuminated with 450 to 490 nm

light.

Transformation of plant cells with a gene encoding GFP and detection of fluorescence has been reported.

Haseloff et al., TIG11: 328-329(1995). Transformed
Arabidopsis cells could be regenerated into whole plants.

However, the regenerated plants expressing GFP exhibited signs of mild to moderate toxicity in the light compared to plants not expressing GFP. The strongest GFP expressors proved more difficult to regenerate.

Likewise, in a recent report describing GFP expression in kanamycin-selected tobacco transformants, Chiu et al.,
Current Biol. 6: 325-330 (1996) note that high GFP expression levels inhibited regeneration of transgenic plants.

A need therefore exists for a cell transformation method which does not rely, or does not rely solely on, selection of cells carrying a gene that confers resistance to a toxic substance. A need exists for a method for efficiently and easily identifying transformed plant cells with a visual screenable marker. A need also exists for a method of cell transformation that does not require destruction of presumptively transformed tissue to assay for the presence of a selectable marker gene.

A need exists for a method for cell transformation that combines a selectable marker gene and a screenable marker gene. In addition, a need exists for a method of cell transformation which does not require exogenous supply of a substrate or cofactor for detection of the polypeptide encoded by a selectable marker gene. Yet another need exists for a method of cell transformation that circumvents the cellular toxicity of the GFP.

SUMMARY OF THE INVENTION
Accordingly, it is an object of the present invention to provide a method for cell transformation which does not depend on selection of cells carrying a gene that confers resistance to a toxic substance.

It is another object of the present invention to provide a method for cell transformation which combines the selection of cells carrying a gene that confers resistance to a toxic substance with screening cells for the presence of a substance that renders transformed cells identifiable.

It is a further object of the present invention to provide a method for cell transformation which does not require destruction of presumptively transformed tissue to assay for the presence of a selectable marker gene.

Yet another object of the present invention is to provide a method for cell transformation which does not require that an exogenous substrate or cofactor be provided to assay for a polypeptide encoded by a selectable marker gene.

It is another object of the present invention to provide a method for cell transformation that circumvents the cellular toxicity of the GFP.

These and other objects are achieved, in accordance with one embodiment of the present invention, by providing an isolated DNA molecule comprising a nucleotide sequence encoding the GFP operably linked to an inducible promoter. The inducible promoter can be selected from the group consisting of the estrogeninducible promoter, the estradiol-inducible promoter, the ACE1 promoter, theIN2 promoter and the tetracycline repressor promoter.

Also provided is an isolated DNA molecule comprising as nucleotide sequence encoding the GFP wherein the nucleotide sequence is operably linked to a targeting sequence for subcellular localization which directs a protein to a subcellular compartment.

An isolated DNA molecule is provided comprising a nucleotide sequence selected from the group consisting of (a) SEQ ID NO: 1; (b) a nucleotide sequence that has substantial sequence similarity with SEQ ID NO: 1; and (c) a functional fragment of (a) or (b), wherein said DNA molecule encodes a GFP. The nucleotide sequence encoding a GFP may be operably linked to a nucleotide sequence encoding a

targeting sequence for subcellular localization which directs a protein to a subcellular compartment. The nucleotide sequence encoding a GFP and a targeting sequence may further comprise a promoter operably linked to said nucleotide sequence.

Also provided are expression vectors comprising DNA molecules encoding a GFP, optionally operably linked to an inducible promoter and/or targeting sequence. The expression vector of the instant invention may carry a nucleotide sequence encoding a foreign protein operably linked to a second promoter.

Also provided is a method of using the expression vectors of the instant invention to produce a transformed plant, comprising the steps of introducing an expression carrying a gene encoding GFP into regenerable plant cells and selecting cells containing the GFP for regeneration.

The method of the instant invention may include the step of inducing GFP expression where the nucleotide sequence encoding GFP is operably linked to an inducible promoter.

The regenerable plant cells utilized in the method of the instant invention are selected from the group consisting of Zea, Brassica and Helianthus cells.

Also provided are transgenic plants expressing the isolated DNA molecule encoding GFP. In addition, transgenic plants comprising a vector carrying a nucleotide sequence encoding GFP are provided.

A method for producing a transgenic plant is provided comprising the steps of (a) constructing an expression vector comprising (i) a first promoter which is an inducible promoter, operably linked to a nucleotide sequence encoding a GFP, and (ii) a second promoter operably linked to a foreign gene; (b) introducing said expression vector into regenerable plant cells; (c) inducing expression of the gene encoding the GFP and selecting transformed plant cells containing said protein; and (d) regenerating transformed plants from said selected transformed plant cells. The inducible promoter may be selected from the group including the estrogen-inducible promoter, the estradiol-inducible promoter, the ACE1 promoter, the IN2 promoter and the tetracycline repressor promoter.

Also provided is a method for producing a transgenic plant, comprising the steps: (a) constructing an expression vector comprising (i) a first promoter operably linked to a nucleotide sequence encoding a sequence for subcellular localization which directs a protein to a subcellular compartment which is operably linked to a nucleotide sequence encoding a GFP, and (ii) a second promoter operably linked to a foreign gene; (b) introducing said expression vector into regenerable plant cells; (c) selecting transformed plant cells containing said GFP; and (d) regenerating transformed plants from said selected transformed plant cells. The targeting sequence for subcellular localization directs the GFP to the mitochondria, chloroplasts, peroxisomes, vacuole, endoplasmic reticulum, cell wall or for secretion generally into the apoplast. This method may include a nucleotide sequence encoding a GFP selected from the group consisting of: (a) SEQ ID NO:l; (b) a nucleotide sequence that has substantial sequence similarity with
SEQ ID NO: 1; and (c) a functional fragment of (a) or (b).

Also provided is a method for producing a transgenic plant, comprising the steps: (a) constructing an expression vector comprising (i) a first promoter operably linked to a nucleotide sequence encoding a screenable marker flanked on the 5-prime and 3-prime ends with a recombinase-specific target sequence, and (ii) a second promoter operably linked to a foreign gene; (b) introducing said expression vector into regenerable plant cells; (c) selecting transformed plant cells containing said screenable marker; (d) transforming the plant cells with a second expression vector containing a gene encoding a site-specific recombinase; (e) selecting plant cells that no longer express the screenable marker; (f) regenerating transformed plants from said selected transformed plant cells; and (g) isolating said foreign protein. The site-specific recombinase may be selected from the group consisting of the FLP/FRT, Ac/DS and cre/lox systems. In addition, the screenable marker may be the GFP.

An isolated DNA molecule comprising a nucleotide sequence encoding the GFP fused in frame to a nucleotide sequence encoding superoxide dismutase is also provided.

Cells transformed with the DNA molecule contain a fusion protein that (i) produces green fluorescence in the presence of UV to blue light and (ii) displays superoxide dismutase activity.

Also provided is a method for producing a transgenic plant comprising: (a) constructing an expression vector comprising (i) a first promoter operably linked to a nucleotide sequence encoding a GFP, and (ii) a

second promoter operably linked to a gene encoding an enzyme that is an oxygen scavenger, and (iii) a third promoter operably linked to a foreign gene; (b) introducing said expression vector into regenerable plant cells; (c) selecting transformed plant cells containing said GFP; and (d) regenerating transformed plants from said selected transformed plant cells. The oxygen scavenger enzyme may be superoxide dismutase.

Alternatively, a method for producing a transgenic plant is provided comprising: (a) constructing an expression vector comprising (i) a first promoter operably linked to a nucleotide sequence encoding a fusion protein comprising the GFP and an oxygen scavenger enzyme fused in frame, and (ii) a second promoter operably linked to a foreign gene; (b) introducing said expression vector into regenerable plant cells; (c) selecting transformed plant-cells containing said the GFP and oxygen scavenger enzyme activity; and (d) regenerating transformed plants from said selected transformed plant cells. The oxygen scavenger enzyme may be superoxide dismutase.

BRIEF DESCRIPTION OF THE DRAWINGS
Figure 1 presents the nucleotide sequence[SEQ ID NO: 1] encoding a GFP with its corresponding amino acid sequence [SEQ ID NO: 2].

Figure 2 shows the Plasmid Map of PHP167.

Figure 3 shows the Plasmid Map of PHP762.

Figure 4 shows the Plasmid Map of PHP7921.

Figure 5 shows the Plasmid Map of PHP8144.

DETAILED DESCRIPTION. 1. Definitions
In the description that follows, a number of terms are used extensively. The following definitions are provided to facilitate understanding of the invention.

A structural gene is a DNA sequence that is transcribed into messenger RNA (mRNA) which is then translated into a sequence of amino acids characteristic of a specific polypeptide.

A promoter is a DNA sequence that directs the transcription of a structural gene. Typically, a promoter is located in the 5-prime region of a gene, proximal to the transcriptional start site of a structural gene. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutivepromoter. For example, a promoter may be regulated in atqasue-flnecific ortissue Preferred manner such that it is only active in transcribing the associated coding region in a specific tissue type(s) such as leaves, roots or meristem.

AcvtoDlasmicalls localized protein is a protein coded for by a gene which does not include any specific signal for targeting of the protein into any subcellular organelle or compartment or any signals for secret ion of the protein. For example, the GFP structural gene operably linked 5-prime to a promoter and to an appropriate 3-prime sequence encodes for a protein compartmentalized in the cytoplasm.

A signal ortargeting sequence is a structural peptide domain required for targeting of a given polypeptide to a subcellular organelle, subcellular compartment or secretion from the cell. As used herein, the phrasesequence for subcellular localization is intended to refer collectively to any form of signal, targeting or retention sequence as defined herein. Signal sequences for a polypeptide directed to the chloroplast or mitochondrion are largely localized to the aminoterminus of the polypeptide. Signal sequences for a polypeptide directed to the glyoxysomes and peroxisomes are largely localized to the carboxy-terminal domains of the polypeptide. Accordingly, targeted transport of the
GFP protein is accomplished by means of chemically joining in proper reading frame, orcerably linking, the nucleotide sequence encoding a signal sequence to the 5prime and/or 3-prime region of the GFP structural gene.

A mitochondriaitargeting sequence facilitates the transport of a protein to a mitochondrial compartment.

Typically, the mitochondrial targeting sequence is located at the amino-terminus of a polypeptide.

A secretiontargeting sequence targets a polypeptide for export into the extracellular space through the

ER.

For example, operably linking a nucleotide sequence encoding the barley alpha amylase 1 (BAA) secretory targeting sequence to the 5-prime end of a structural gene targets the encoded protein for export into the extracellular space.

A cell walltargeting sequence targets a polypeptide for export from the cell but the polypeptide is specifically localized to the cell wall. For example, cell wall localization of a polypeptide is accomplished by operably linking a nucleotide sequence encoding BAA 5prime, and operably linking a nucleotide sequence encoding a portion of the maize hydroxyproline-rich glycoprotein 3-prime to a gene encoding the polypeptide.

Steifel et al., Plant Cell 2: 785-793 (1990).

A vacuolar sianal sequence facilitates the transport of a protein to the vacuole. For example, vacuolar targeting is accomplished by fusing the BAA secretory signal sequence at the amino-terminus of the protein and a sequence encoding a vacuolar signal sequence to the carboxy-terminus. Transport of a polypeptide to the vacuole is therefore accomplished by means operably linking a nucleotide sequence encoding BAA 5-prime, and a nucleotide sequence encoding a vacuolar signal sequence 3-prime to a gene encoding a polypeptide. Alternatively, vacuolar targeting is accomplished by constructing a nucleotide sequence comprising in the 5-prime to 3-prime direction nucleotide sequences encoding a vacuole signal sequence, BAA and a polypeptide.

An endoplasmic reticulum retention sequence targets a polypeptide for localization in the lumen of the endoplasmic reticulum. For example, a polypeptide is targeted for retention in the endoplasmic reticulum through the addition of the BAA sequence on the aminoterminus and an endoplasmic reticulum signal sequence on the carboxy-terminus of the polypeptide.

A nucleartargeting sequence facilitates transport of a polypeptide to the nucleus. Typically, the nuclear signal sequence is located at the amino-terminus of a polypeptide. In order to retain the nuclear targeted protein in the nucleus, it may be necessary to increase the molecular weight of the protein by means of fusing an unrelated protein to the carboxy-terminus of the targeted protein. For example, GFP was retained in the nucleus by operably linking a nucleotide sequence encoding a nuclear signal sequence 5-prime and a nucleotide sequence encoding maize acetolactate synthase 3-prime to a gene encoding a polypeptide.

ADeroxisonal targeting sequence facilitates the transport of a polypeptide into the peroxisome.

Typically, the peroxisomal signal sequence is a tripeptide located at the carboxy-terminus of a polypeptide.

AchloroPlast taraetgna sequence facilitates the transport of a nuclear encoded protein to a chloroplast compartment. Typically, the chloroplast signal sequence is located at the amino-terminus of a polypeptide.

Accordingly, transport of a polypeptide to a chloroplast compartment is accomplished by means of operably linking the nucleotide sequence encoding a chloroplast signal sequence to the 5-prime region of a gene encoding a polypeptide.

An isolated DNA molecule is a fragment of DNA that is not integrated in the genomic DNA of an organism.

Co=lementarv DNA (cDNA) is a single-stranded DNA molecule that is formed from an mRNA template by the enzyme reverse transcriptase. Typically, a primer complementary to portions of mRNA is employed for the initiation of reverse transcription. Those skilled in the art also use the term"cDNA" to refer to a doublestranded DNA molecule consisting of such a singlestranded DNA molecule and its complementary DNA strand.

The term expression refers to the biosynthesis of a gene product. For example, in the case of a structural gene, expression involves transcription of the structural gene into mRNA and the translation of mRNA into one or more polypeptides.

A cloning vector is a DNA molecule, such as a plasmid, cosmid, or bacteriophage, that has the capability of replicating autonomously in a host cell.

Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites at which foreign DNA sequences can be inserted in a determinable fashion without loss of an essential biological function of the vector, as well as a marker gene that is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include, for example, genes that provide tetracycline resistance, ampicillin resistance or kanamycin resistance.

An expression vector is a DNA molecule comprising a gene that is expressed in a host cell. Typically, gene expression is placed under the control of certain regulatory elements, including constitutive or inducible promoters, tissue-specific regulatory elements, and enhancers. Such a gene is said to be operably linked to the regulatory elements.

Transformation includes introduction of genetic material into plant cells resulting in chromosomal integration and stable heritability through meiosis.

Transformation also includes introduction of genetic material into plant cells in the form of plant viral vectors involving epichromosomal replication and gene expression which may exhibit variable properties with respect to meiotic stability.

A foreignsene refers in the present description to a DNA sequence that is operably linked to at least one heterologous regulatory element.

A recombinant host may be any prokaryotic or eukaryotic cell that contains either a cloning vector or expression vector. This term also includes those prokaryotic or eukaryotic cells that have been genetically engineered to contain the cloned gene(s) in the chromosome or genome of the host cell.

Atransenic plant is a plant having one or more plant cells that contain an expression vector.

A preen fluorescent Protein (GFP), or a functional fragment of a GFP, is capable of producing a green fluorescence. GFP absorbs in the W to blue range with a peak at 395 nm and emits in the green with a peak at 510 nm. The "red-shifted" version of GFP is a modified version of GFP which absorbs between 480 to 490 nm and emits in the green with a peak at 510nm. Red-shifted GFP is abbreviated as GFPr. The "blue fluorescent protein" is a modified version of GFP which absorbs around 380nm and emits in the blue with a peak at around 445nm. Blue fluorescent protein is abbreviated as BFP. GFPm is a nucleotide sequence coding for GFP in which the DNA sequence has been modified based on codon preference in maize (Fig. 1).

Two nucleic acid molecules are considered to have a substantialso once similarity if their nucleotide sequences share a similarity of at least50k. Sequence similarity determinations can be performed, for example, using the FASTA program (Genetics Computer Group; Madison,WI). Alternatively, sequence similarity determinations can be performed using BLAST (Basic Local
Alignment Search Tool) of the Experimental GENIFO(R)
BLAST Network Service. See Altschul et al., J. Mol.

ilicl. 215: 403(1990). Also, see Pasternak et al.,sequence Similarity Searches, Multiple Sequence Alignments, and Molecular Tree Building," in METHODS IN
PLANT MOLECULAR BIOLOGY AND BIOTECHNOLOGY, Glick et al.

.eds.), pages 251-267 (CRC Press 1993).

2. Reducing GFP Toxicity in Transformed Plants
Oxidative stress associated with GFP fluorescence has been suggested to cause cellular toxicity. Haseloff et al., TIG 11: 328-329 (1995). If GFP is to be useful as a screenable marker for plant transformation, as well as a reporter gene for detection of gene expression, strategies for reducing GFP toxicity must be available.

The gene encoding GFP can be operably linked to an inducible promoter so that GFP can be transiently expressed and transformed cells identified.

Alternatively, the gene encoding GFP can be operably linked to a signal sequence for targeting to an organelle or subcellular compartment or for secretion to the apoplast where GFP is not toxic to the cell.

Oxidative stress can be ameliorated by transforming cells containing GFP with an enzyme that serves as an oxygen scavenger. Such enzymes are well known in the art. For example, genes encoding the enzyme superoxide dismutase may protect against the primary oxidative stresses associated with GFP fluorescence. Balzan et al., Proc. Nat Acad. Sci. 92: 4219-4223 (1995). Other enzymes involved in the oxidative stress response, such as ascorbate peroxidase or glutathione5-transferase, could help mitigate secondary effects in the cell.

Conklin, Plant Physiol. 109: 203-212 (1995).

More specifically, oxidative stress can be ameliorated by transforming cells with a DNA construct carrying genes encoding GFP and SOD. Alternatively, the gene encoding GFP can be fused in frame to the gene encoding SOD. Cells transformed with this DNA construct produce a fusion protein that cause the cells to fluoresce in the presence of UV-blue light and express SOD activity.

The toxicity of GFP in transformed plants can be eliminated by excising the screenable marker gene following detection of transformed cells or sectors. The FLP/FRT system is used in conjunction with GFPm as a visible viable marker for FRT excision. The FLP/FRT system has been demonstrated in maize suspension cells using GUS expression as an indicator of FRT excision.

Lysnik et.al., NAR21: 969-975 (1993). For example, plant cells are bombarded with a DNA construct containing the GFP gene flanked by FRT sequences as well as a foreign or agronomic gene of interest. The GFP gene may be operably linked to a constitutive promoter or an inducible promoter. In addition, the GFP gene may be operably linked to a signal sequence. Stable transformants are detected by means of screening for GFP.

Transgenic callus pieces are spread on medium and bombarded a second time with a FLP recombinase construct.

Callus is monitored periodically under UV to blue illumination to detect cells that no longer express GFP.

Callus pieces that no longer express GFP are regenerated and analyzed for expression of the foreign or agronomic gene. Agronomically useful transgenic plants are thereby produced that do not contain a marker gene.

The sensitivity of the screening method can be further increased by placing two markers genes between the FRT sequences. For example, plant cells are bombarded with a DNA construct containing the PAT and GFP genes flanked by FRT sequences and a foreign or agronomic gene. Stable transformants are recovered on bialaphoscontaining medium and positive GFP-expression is confirmed. The transformed callus is then bombarded with FLP. The callus is then grown for 2-6 weeks with no selection until clear GFP-null sectors can be identified.

These sectors can be transferred onto bialaphos/chlorophenol red multiwell test plates to confirm bialaphos sensitivity (i.e. within 3-5days).

Callus pieces that no longer express GFP or PAT are regenerated and analyzed for expression of the foreign or agronomic gene. This permits recovery of agronomically useful transformants without any marker genes in the final product.

Likewise, the Ac/Ds system of maize can also be used in transgenic plants to excise the screenable marker gene that is transformed together with a foreign or agronomic gene. Mobilization of Ac and/or Ds has been demonstrated in diverse plants such as tomato, tobacco and Arabidopsis. Yoder et al., In Tomato Technology, Alan R.

Liss, Inc. pp 189-198 (1987); Yoder et al., U.S. Patent No. 5,225,341; Baker et al., EMBO J 6: 1547-1554 (1987) and Lawson et al. Mol. Gen. Genet., 245: 608-615 (1994).

Likewise, the cre/lox recombinase system from bacteriophage P1 could also be used in conjunction with GFP. Excision of transgenes in plants using the cre/lox system was first demonstrated in tobacco. Odell et al.,
Mol. Gen. Genet., 223: 369-378 (1990) and Dale and Ow,
Proc. Natl. Acad. Sci. USA, 88: 10558-10562 (1991)
Similar to the FLP and Ac systems described above, GFP expression provides an efficient, easily scorable phenotype for monitoring excision.

3. Isolation of Nucleotide Sequences Encoding GFP
Peptide sequences from purified GFPs are used to design degenerate oligonucleotide primers for polymerase chain reactions and gene cloning. Protein extracts can be prepared from cnidarian cells by standard methods known to the art. See, for example, Harlow and Lane,
Antibodies, A Laboratory Manual, Cold Spring Harbor
Press,(1988). In a preferred embodiment, cnidarian cells are extracted into a buffer, and the extracts separated into membrane and soluble fractions. Each fraction is tested for green fluorescence activity in the presence of blue light.

Fractions which contain GFP activity are then purified further to determine which components are responsible for the activity. Purification of the active fractions can be carried out by methods known in the art.

See, for example, PROTEIN PURIFICATION METHODS - A
PRACTICAL APPROACH, Harris et al., Eds. (IRL Press,
Oxford, 1989). Extracts prepared as described above are purified by sequential size exclusion chromatography iscelectric focusing, HPLC size exclusion chromatography, and chromatography on an affinity column. Fractions which display GFP activity can be analyzed further by
SDS-PAGE analysis to determine the approximate molecular mass of the active component.

Purified GFPs prepared by the methods described above can be sequenced using methods well known in the art, for example using a gas phase peptide sequencer (Applied Biosystems, Foster City, CA). To determine as much of the peptide sequence as possible, it is preferred that the proteins of the present invention be cleaved into smaller fragments more suitable for gas-phase sequence analysis. This can be achieved by treatment of a purified GFP with selective peptidases, and in a particularly preferred embodiment, with endoproteinase lys-C (Boehringer). The fragments so produced can be separated by reversed-phase HPLC chromatography.

The peptide sequences of the proteins determined as above can be used to determine the DNA sequence encoding the protein. Methods for carrying out this determination are well known in the art. See, for example Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition, (Cold Spring Harbor Laboratory Press, Cold
Spring Harbor, NY 1989).

In a preferred embodiment of the present invention, the peptide sequences are used to design degenerate oligonucleotide primers for polymerase chain reactions.

Each degenerate primer set will preferably contain every possible DNA sequence encoding the corresponding peptide sequences. Primer sets are prepared in both the sense and antisense orientation. Suitable oligonucleotide primers can be synthesized using commercial synthesizers, such as those supplied by Applied Biosystems (Foster
City, CA). In a particularly preferred embodiment, the primers include additional nucleotide sequences containing restriction endonuclease cleavage sites. The presence of such sites allows for the directional cloning of PCR products into suitable cloning vectors after treatment with an appropriate restriction enzyme. See
Finney, "Molecular Cloning of PCR Products" in CURRENT
PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel et al. Eds. (John
Wiley & Sons, New York,1987) p. 15.7.1.

Template DNA for the PCR can be prepared from appropriate cnidarian cells or tissues using methods well known in the art. See Sambrook et al., supra. In a preferred embodiment, host cells are crushed under liquid nitrogen and mRNA is extracted using a commercially available kit (Pharmacia, Piscataway, NJ).

The mRNA preparation can then be used as a template for cDNA synthesis usingpoly(dT) or random hexamer primers by standardtechniques. See Sambrook et al., supra. In a particularly preferred embodiment, cDNA synthesis is carried out using a commercially available kit (Pharmacia).

The cDNA can then be used directly for PCR using the method of Saiki et al., Science 239: 487 (1988). The cDNA also is used to prepare a cDNA library by standard methods. See Sambrook et al., supra. In a particularly preferred embodiment, the cDNA is packaged into bacteriophage particles using a commercially available kit (Promega, Madison, WI). The packaged cDNA is then transfected into E. coli to produce a cDNA library.

In an alternative preferred embodiment, genomic DNA from cnidarian cells or tissue can be used as the template DNA for the PCR. Genomic DNA can be prepared by standard methods and PCR can then be used to prepare double stranded DNA molecules to probe the cDNA library and the genomic DNA for the gene(s) encoding GFP. In a preferred embodiment, degenerate primers are prepared corresponding to the termini of the longest peptide sequence determined by peptide sequencing. In a particularly preferred embodiment, primers are used in a
PCR with first strand cDNA as template to amplify the DNA encoding the peptide. PCR is carried out under standard conditions. See Sakai et al., supra.

PCR amplification products are analyzed by polyacrylamide gelelectrophoresis using standard methods. If an amplification product of the expected size (based on the peptide sequence) is found, the product is digested with appropriate restriction enzymes, ligated into a cloning vector and cloned by standard methods. See Sambrook et al, supra. In a preferred embodiment, clones are sequenced to verify that sequences according to the expected peptide sequence are present.

Once the DNA sequence encoding the peptide is known, it can be used to prepare non-degenerate primers corresponding to that sequence, again containing restriction enzyme recognition sequences to aid in cloning of DNA products. These primers are used in combination with degenerate primers corresponding to other peptide sequences to generate PCR amplification products which can be cloned and then analyzed as above.

By these means, fragments of the gene sequence of the protein can be determined. Alternative methods for carrying out this PCR analysis include use of the 5' or 3' RACE methods using commercially available kits, such as those manufactured by Life Technologies (Gaithersburg,
MD) or Clontech (Palo Alto, CA). Primers for this method are selected according to the manufacturer's directions.

Gene fragments prepared as above are excised from the cloning vector by restriction enzyme digestion, labeled with32P by conventional methods and used as probes to identify the complete gene encoding the protein from within a cDNA or genomic library. In a preferred embodiment, the probe is chosen such that it is long enough to ensure hybridization specificity, while remaining short enough to allow reasonable rates of hybridization to the target gene.

A cnidarian genomic DNA library can be prepared by means well-known in the art. See, for example, Slightom et al. "Construction of X Clone Banks," in METHODS IN
PLANT MOLECULAR BIOLOGY AND BIOTECHNOLOGY, Glick et al.

(eds.), pages 121-146 (CRC Press, 1993). Genomic DNA can be isolated from cnidarian tissue, for example, by lysing plant tissue with the detergent Sarkosyl, digesting the lysate with proteinase K, clearing insoluble debris from the lysate by centrifugation, precipitating nucleic acid from the lysate using isopropanol, and purifying resuspended DNA on a cesium chloride density gradient.

See, for example, Ausubel et al. (eds.), CURRENT
PROTOCOLS IN MOLECULAR BIOLOGY, pages 2.3.1-2.3.3 (Wiley
Interscience 1990)["Ausubel"]
DNA fragments that are suitable for the production of a genomic library can be obtained by the random shearing of genomic DNA or by the partial digestion of genomic DNA with restriction endonucleases. See, for example, Ausubel at pages 5.3.2-5.4.4, and Slightom et al., supra. Genomic DNA fragments can be inserted into a vector, such as a bacteriophage or cosmid vector, in accordance with conventional techniques, such as the use of restriction enzyme digestion to provide appropriate termini, the use of alkaline phosphatase treatment to avoid undesirable joining of DNA molecules, and ligation with

appropriate ligases. Techniques for such manipulation are disclosed by Glightom et al., supra, and are well-known in the art. Also see Ausubel at pages 3.0.5-3.17.5.

Screening of the cDNA or genomic library is carried out by conventional methods. See Sambrook et al, supra.

Clones which hybridize to the probe are purified and their sequences determined. To facilitate sequencing, nested deletions in the clones can be created using standard protocols, or by commercially available kits such as Erase-a-base (Promega, Madison, WI) or The
Deletion Factory (Life Technologies, Gaithersburg, MD), following the manufacturer's instructions. The sequences obtained are analyzed for the presence of open reading frames by conventional methods and to check if the entire gene sequence has been found. In a preferred embodiment, cDNA libraries are prepared by both random hexamer and poly (dT) priming from samples, and are used to maximize the chances of finding the complete coding sequence of the desired gene.

Once the entire coding sequence of the gene for GFP has been determined, the gene can be inserted into an appropriate expression system. The gene can be expressed in any number of different recombinant DNA expression systems to generate large amounts of protein, which can then be purified.

4. Chemical Synthesis of Genes Encoding GFP
Once the amino acid sequence of a GFP is known, a gene encoding this GFP can be synthesized. In addition, the nucleotide sequence ofn synthetic gene encoding GFP can be optimized for expression in plants by modifying the codon usage to include plant preferred codons. See, for example, Murray et al., NAR 17: 477(1989). Even more specifically, the nucleotide sequence of a synthetic gene encoding GFP can be optimized for expression in monocotyledonous or dicotyledonous plants. See, for example, Campbell et al., Plant Physiol. 92: 1 (1990).

Genes encoding GFP can be obtained, for example, by synthesizing the genes with mutually priming long oligonucleotides. See, for example, Ausubel at pages 8.2.8 to 8.2.13. Also, see Wosnick et al., Gene 60:115(1987) . Moreover, current techniques using the polymerase chain reaction provide the ability to synthesize genes as large as 1.8 kilobases in length.

Adang et al., Plant Molec. Biol. 21: 1131 (1993); Bambot et al., PCR Methods and Applications 2: 266 (1993).

5. Identification of Functional Fragments of the GFP
DNA clones can be analyzed using a variety of techniques such as restriction analysis, Southern analysis, primer extension analysis, and DNA sequence analysis. Primer extension analysis or S1 nuclease protection analysis, for example, can be used to localize the putative start site of transcription of the cloned gene. Ausubel at pages 4.8.1-4.8.5; Waimsley et al., "Quantitative and Qualitative Analysis of Exogenous Gene
Expression by the S1 Nuclease Protection Assay," in
METHODS IN MOLECULAR BIOLOGY, VOL. 7: GENE TRANSFER AND
EXPRESSION PROTOCOLS, Murray (ed.), pages 271-281 (Humana
Press Inc. 1991). Functional fragments of the GFP protein are identified by production of green fluorescence in the presence of blue W light.

The general approach of such functional analysis involves subcloning DNA fragments of a genomic clone, cDNA clone or synthetic gene sequence into an expression vector, introducing the expression vector into a heterologous host, and screening to detect green fluorescence in the presence of W to blue light.

Methods for generating fragments of a cDNA or genomic clone are well-known. Variants of an isolated DNA encoding GFP can be produced by deleting, adding and/or substituting nucleotides for the isolated nucleotides, for example, the nucleotide sequence of SEQ ID NO: 1.

Such variants can be obtained, for example, by oligonucleotide-directed mutagenesis, linker-scanning mutagenesis, mutagenesis using the polymerase chain reaction, and the like. See, for example, Ausubel, pages 8.0.3-8.5.9. Also see generally, McPherson (ed.),
DIRECTED MUTAGENESIS: A PRACTICAL APPROACH, (IRL Press 1991). Thus, the present invention also encompasses DNA molecules comprising nucleotide sequences that have substantial sequence similarity with SEQ ID NO: 1 and encode a GFP.

6. Methods for Plant Transformation

The GFP-based screening method for plant transformation of the instant application can be used in conjunction with any method of plant transformation and regeneration. Numerous methods for plant transformation have been developed, including biological and physical, plant transformation protocols. See, for example, Miki et al., "Procedures for Introducing Foreign DNA into

Plants" in Methods in Plant Molecular Biology and

Biotechnology, Glick, B.R. and Thompson, J.E. Eds. (CRC

Press, Inc., Boca Raton, 1993) pages 67-88. In addition, expression vectors and in vitro culture methods for plant cell or tissue transformation and regeneration of plants are available. See, for example, Gruber et al., "Vectors for Plant Transformation" in Methods in Plant Molecular

Biology and Biotechnology, Glick, B.R. and Thompson, J.E.

Eds. (CRC Press, Inc., Boca Raton, 1993) pages 89-119.

A.Agrobacterium-mediated Transformation

The most widely utilized method for introducing an expression vector into plants is based on the natural transformation system of Agrobacterium. See, for example, Horsch et al., Science 227:1229 (1985). A.

tumefaciens and A. rhizogenes are plant pathogenic soil bacteria which genetically transform plant cells. The Ti and Ri plasmids of A. tumefaciens and A. rhizogenes, respectively, carry genes responsible for genetic transformation of the plant. See, for example, Kado,C.I., Crit. Rev. Plant. Sci. 10: 1 (1991). Descriptions ofAgrobacterium vector systems and methods for

Agrobacterium-mediated gene transfer are provided by

Gruber et.al., supra, Miki et.al., supra, and Moloney et.al., Plant Cell Reports 8: 238 (1989).

B. Direct Gene Transfer

Despite the fact the host range for Agrobacteriummediated transformation is broad, some major cereal crop species and gymnosperms have generally been recalcitrant to this mode of gene transfer, even though some success has recently been achieved in rice. Hiei et al., The

Plant Journal 6: 271-282 (1994). Several methods of plant transformation, collectively referred to as direct gene transfer, have been developed as an alternative to

Agrobacterium-mediated transformation.

A generally applicable method of plant transformation is microprojectile-mediated transformation wherein DNA is carried on the surface of microprojectiles measuring 1 to 4 m. The expression vector is introduced into plant tissues with a biolistic device that accelerates the microprojectiles to speeds of 300 to 600 m/s which is sufficient to penetrate plant cell walls and membranes. Sanford et al., Part. Sci. Technol. 5: 27 (1987), Sanford, J.C., Trends Biotech. 6: 299 (1988)

Sanford, J.C., Physiol. Plant 79: 206 (1990), Klein et al., Biotechnology10: 268 (1992).

Another method for physical delivery of DNA to plants is sonication of target cells. Zhang et al.,Bio/Technology 9: 996(1991)~. Alternatively, liposome or spheroplast fusion have been used to introduce expression vectors into plants. Deshayes et al., EMBO J.,: 2731 (1985), Christou et al., Proc Natl. Acad. Sci. U.S.A. 84: 3962 (1987). Direct uptake of DNA into protoplasts usingCaCl2 precipitation, polyvinyl alcohol or poly-L-ornithine have also been reported. Hain et al., Mol.

Gen. Genet. 199: 161 (1985) and Draper et al., Plant Cell

Physiol. 23: 451 (1982). Electroporation of protoplasts and whole cells and tissues have also been described.

Donn et al., In Abstracts of VIIth International Congress on Plant Cell and Tissue Culture IAPTC, A2-38, p 53 (1990); D'Hailuin et al., Plant Cell 4: 1495-1505 (1992) and Spencer et al., Plant Mol. Biol. 24: 51-61 (1994).

A preferred method is microprojectile-mediated bombardment of immature embryos. The embryos can be bombarded on the embryo axis side to target the meristem at a very early stage of development or bombarded on the scutellar side to target cells that typically form callus and somatic embryos. Targeting of the scutellum using projectile bombardment is well known to those in the art of cereal tissue culture. Klein et al., Bio/Technol., 6: 559-563 (1988); Kartha et al., Plant Cell Rep. 8: 429-432 (1989); Sautter et al., Bio/Technol., 9: 1080-1085 (1991); Chibbar et al., Genome, 34: 435-460 (1991). The scutellar origin of regenerable callus from cereals is well known. Green et al., Crop Sci., 15: 417-421 (1975);

Lu et al., TAG 62: 109-112 (1982); and Thomas and Scott,
J. Plant Physiol.ill: 159-169 (1985). Targeting the scutellum and then using chemical selection to recover transgenic plants is well established in cereals.

D/Halluin et al., Plant Cell 4: 1495-1505 (1992); Perl et al., MGG235: 279-284 (1992); Cristou et al., Bio/Technol. 9: 957-962 (1991). This literature reports
DNA targeting of the scutellum and recovery of transgenic callus, plants and progeny based on a chemical selection regime. None of these references teach successful plant transformation wherin transformed cells are: visualized with a screenable marker such as GUS.

A preferred transformation method involves bombardment of the scutellar surface of immature embryos to introduce a fGP expression cassette and any other cotransformed genes. Embryos can be pretreated for 1 to48 hours with a high osmoticum medium or left on a highosmoticum medium for 24-48 hours after bombardment to improve cell survival and transformation frequencies.

Immature embryos are then cultured on typical callusinducing medium with no selective agent. At each subculture transfer, i.e., every two weeks, the culture is monitored using W-blue light for GFP fluorescence.

Fluorescing calli are separated from non-fluorescing callus, and grown to the point where plants can be regenerated through standard media progressions.

Many exemplary variations, in terms of target cells and tissue culture routes are known in the art that could be used with GFP in this fashion. Some of these variations include introduction of GFP expression into protoplasts, suspension cells, type II callus, and type
I callus, as in Morocz et al., Theor. Appl.Genet. 80: 721-726 (1990), Gordon-Kamm et.al., Plant Cell 2: 603-618 (1990), Fromm.et al:,Bio/Technol. 8: 833-839 (1990) and
D'Halluin et.al., Plant Cell 4: 1495-1505 (1992).

Microspores and microspore-derived embryogenic callus also represent a feasible alternative target/culturing route for using GFP screening to recover transformants.

See Sun et.al., Plant Cell Rep. 8: 313-316 (1989) and
Mitchell et.al., J. Plant Physiol. 37: 530-536 (1991).

Furthermore, it is expected that the method of the present invention is also be applicable to any dicot species that is known to have a callus stage, for example, tobacco, canola or soybean.

In the case of the maize meristem target, the method entails selective enlargement of transgenic sectors, toward genetic homogeneity, in cell layers that contribute to germline transmission. This method is described in copending U.S. patent application 08/483,091, incorporated herein by reference, and comprises the steps of (A) introducing foreign DNA into a meristem that is not enclosed by sheathing leaves; (B) inducing reorganization of the meristem to increase transgenic sector size, whereby the likelihood that a transgenic sector will contribute to germline transmission is increased; and (C) exposing the meristem to conditions under which it differentiates to form a plantlet, wherein the plantlet contains the transgenic sector or is homogeneously transformed by the foreign
DNA, such that the plant let can be grown into a transformed cereal plant that will transmit the foreign DNA to progeny. The foreign DNA can be introduced into a plurality of meristems, at least some of which differentiate in step (C) to form a plurality of plantlets.

In one preferred embodiment, reorganization is effected through at least one manipulation selected from the group consisting of (i) imposition of a nonlethal selective pressure on the meristems, (ii) mechanicallyinduced meristem reorganization, and (iii) hormonallyinduced shoot multiplication. In another preferred embodiment the conditions in step (C) are such that the meristems undergo maturation and plant differentiation to form shoot apices, and the method further comprises effecting reorganization of meristem tissue in the shoot apices to enlarge transformed sectors or to produce periclinal L2 chimera other DNA delivery methods can be used with this explant such as bombardment with DNA-coated particles, or silicon carbide fiber-mediated delivery. Kaeppler et al., Plant
Cell Rep. 9: 415-418(1990) . After DNA introduction, the meristem explants are cultured.

Plants can be manipulated, for example, by removal of the apical meristem, to stimulate axillary or secondary buds which can exhibit larger transgenic sectors relative to the primary shoot. Flowers above

transgenic shoots are pollinated and the progeny are analyzed for transgene presence and expression. A variety of starting explants can regenerate shoots in sunflower, and thus represent alternative targets for GFP-encoding DNA delivery and transmission to progeny.

These include the seedling meristem (as above), also the seedling hypocotyl, the mature cotyledon, the immature cotyledon, zygotic immature embryos, somatic embryos, and primary leaflets. See for example, respectively, Greco et al., Plant Sci. Lett. 36:73-77 (1984); Krauter et al., Helia 14: 117-122 (1991); Power Am. J. Bot. 74:497- 503 (1987); Krauter et al., Theor. Appl. Genet.82: 521525 (1991); Finer, Plant Cell Rep. 6: 372-374 (1987), and
Greco et al., Plant Sci. Lett. 36: 73-77 (1984).

In similar fashion, GFP expression could be used in other dicot species, for example, soybean, to map sectors and/or identify transformants and follow the transgenes through to progeny. A GFP-encoding cassette and other cotransformed genes can be introduced into soybean cotyledonary node cells using Agrobacterium. The explants are cultured on Gamborg (B5) medium with or without kanamycin selection. With no selection, GFP expression is used to identify transgenic chimeric or homogeneously transformed plantlets, which are rooted, grown to maturity and pollinated in the greenhouse.

Progeny seed are collected for analysis. The use of cotyledonary nodes and Agrobacterium is presented by way of example, but other target cells and culturing methods could be used with GFP for soybean. In addition to the cotyledonary node cells, mature cotyledons and immature cotyledons have both been used for transformation. See
Hinchee et al.,Bio/Technol. 6: 915-922 (1988) and
Parrott et al., Plant Cell Rep. 7: 615-617 (1989).

Meristems from immature soybean seed embryonic axes have also been used for bombardment-mediated soybean transformation. See Christou et al., Plant Physiol. 87: 671-674 (1988) and McCabe et al.,Bio/Technol. 6: 923-926 (1988).

7. Promoters .
A. Inducible Promoters
An inducible promoter is operably linked to a nucleotide sequence encoding a GFP. Optionally, the inducible promoter is operably linked to a nucleotide sequence encoding a signal sequence which is operably linked to a nucleotide sequence encoding GFP. With an inducible promoter the rate of transcription increases in response to an inducing agent.

Any inducible promoter can be used in the instant invention. See Ward et al. Plant Mol. Biol. 22: 361-366 (1993). Exemplary inducible promoters include that from the ACE1 system which responds to copper (Mett et al.

PNAS 90: 4567-4571 (1993));In2 gene from maize which responds to benzenesulfonamide herbicide safeners (Hershey et al., Mol. Gen. Genetics 227: 229-237 (1991) and Gatz et al., Mol. Gen. Genetics 243: 32-38 (1994)) or
Tet repressor from TnI0 (Gatz et al., Mol. Gen. Genet.

227: 229-237 (1991). A particularly preferred inducible promoter is a promoter that responds to an inducing agent to which plants do not normally respond. An exemplary inducible promoter is the inducible promoter from a steroid hormone gene thetranscriptional activity of which is induced by a glucocorticosteroid hormone.

Schena et al., Proc. Natl. Acad. Sci.U.S.A. 88: 10421(1991)
The expression vector comprises an inducible promoter operably linked to a nucleotide sequence encoding GFP. The expression vector is introduced into plant cells and presumptively transformed cells are exposed to an inducer of the inducible promoter. The cells are screened for the presence of GFP protein by means of illuminating the cells with W to blue light and screening for the presence of green fluorescence.

B.Tissue-specific or Tissue Preferred Promoters
A tissue-specific promoter is operably linked to a nucleotide sequence encoding a GFP. Optionally, the tissue-specific promoter is operably linked to a nucleotide sequence encoding a signal sequence which is operably linked to a nucleotide sequence encoding GFP.

Plants transformed with a gene encoding GFP operably linked to a tissue-specific promoter produce the GFP protein exclusively, or preferentially, in a specific tissue.

Any tissue-specific or tissue-preferred promoter can be utilized in the instant invention. Exemplary tissuespecific or tissue-preferred promoters include a rootpreferred promoter such as that from the phaseolin gene (Murai et al., Science23:~476-482 (1983) and Sengupta
Gopalan et al., Proc. Natl. Acad. Sci. USA 82: 3320-3324 (1985)); a leaf-specific and light-induced promoter such as that from cab or rubisco (Simpson et al., EMBO J.

4(11): 2723-2729 (1985) and Timko et al., Nature 318: 579-582(1985)); an anther-specific promoter such as that fromLAT52 (Twell et al., Mol. Gen. Genet. 217: 240-245 (1989)); a pollen-specific promoter such as that from
Zml3 (Guerrero et al., Mol. Gen. Genet. 224: 161-168 (1993)) or a microspore-preferred promoter such as that from apg (Twell et al., Sex. Plant Reprod. 6: 217-224(1993).

The expression vector comprises a tissue-specific or tissue-preferred promoter operably linked to a nucleotide sequence encoding GFP. The expression vector is introduced into plant cells. The cells are screened for the presence of GFP protein by means of illuminating the cells with UV to blue light and screening for the presence of green fluorescence.

C. Constitutive Promoters
A constitutive promoter is operably linked to a nucleotide sequence encoding a GFP or the constitutive promoter is operably linked to a nucleotide sequence encoding a signal sequence which is operably linked to a nucleotide sequence encoding GFP.

Many different constitutive promoters can be utilized in the instant invention. Exemplary constitutive promoters include the promoters from plant viruses such as the35S promoter rom CaMV (Odell et al., Nature 313: 810-812 (1985) and the promoters from such genes as rice actin (McElroy et al., Plant Cell 2: 163171 (1990)); ubiquitin (Christensen et al., Plant Mol.

Biol. 12: 619-632 (1989) and Christensen et al., Plant
Mol. Biol.18: 675-689 (1992)); pEMU (Last et al., Theor.

App7. Genet. 81: 581-588 (1991)); MAS (Velten et al.,
EMBO J. 3: 2723-2730 (1984)) and maize H3 histone (Lepetit et al., Mol. Gen. Genet. 231: 276-285 (1992) and
Atanassova et al., Plant Journal 2(3): 291-300 (1992)).

The ALS promoter, a Xbal/Ncol fragment 5-prime to theBrassica napus ALS3 structural gene (or a nucleotide sequence that has substantial sequence similarity to said
Xbal/Ncol fragment), represents a particularly useful constitutive promoter. Co-pending Pioneer Hi-Bred International US Patent Application 08/409,297.

The expression vector comprises a constitutive promoter operably linked to a nucleotide sequence encoding GFP. The expression vector is introduced into plant cells and presumptively transformed cells are screened for the presence of GFP protein by means of illuminating the cells with UV to blue light and screening for the presence of green fluorescence.

8. Signal Sequences For Targeting Proteins to
Subcellular Compartments
Transport of GFP to a subcellular compartment such as the chloroplast, vacuole, peroxisome, glyoxysome, cell wall or mitochondrion, or for secretion into the apoplast, is accomplished by means of operably linking the nucleotide sequence encoding a signal sequence to the 5-prime and/or 3-prime region of a gene encoding GFP.

Targeting sequences at the S-prime and/or 3-prime end of the structural gene may determine, during protein synthesis and processing, where the encoded protein is ultimately compartmentalized. The presence of a signal sequence directs a polypeptide to either an intracellular organelle or subcellular compartment or for secretion to the apoplast.

In addition to the targeting sequences described above, the addition of amino acids to the encoded protein (fusing GFP to either another protein, a protein fragment, or a peptide) can further influence the

fate of
GFP. For example, a nuclear localization signal (NLS) alone is not sufficient to accumulate GFP in the nucleus.

The GFP protein is sufficiently small that it freely diffuses from the nucleus into the cytoplasm, probably through the nuclear pores. The fusion of GFP to a sufficiently large protein permits accumulation in the nucleus if the GFP protein also contains an NLS at the amino-terminus. GFP can also be targeted to the nucleus by fusing GFP to proteins that are normally targeted to the nucleus.

The following exemplary signal sequences were utilized for targeting of GFP. However, the invention encompasses use of any signal sequence, or combination of signal sequences, to facilitate transport of GFP to a subcellular compartment or the apoplast where the protein is not toxic to the cell but can be detected.

More specifically, GFP is expressed in plant cells transformed with a expression vector carrying a nucleotide sequence encoding a signal sequence(s), which directs a protein to a subcellular compartment or to the apoplast, operably linked to a gene encoding GFP. This GFP gene construct can be expressed from a constitutive, tissue-specific, tissue-preferred or inducible promoter.

GFP is transported to a subcellular compartment or the apoplast where it is not toxic to the plant cell. Cells illuminated with W to blue light can be detected because they emit green fluorescence. The transformed cells are selected for regeneration. If the expression vector also carries a gene encoding a foreign protein or agronomically useful protein, the regenerated plant can be used for production of this protein.

A. PHPSO8O - Ubi::CTP-GFPm
A chloroplast signal sequence from the maize cab-m7 has been characterized and the nucleotide sequence is shown below. Becker et al., Plant Mol. Biol. 20: 49 (1992). The signal sequence includes 21 amino acids of the cab-m7 protein that extends beyond the processing site (marked by *).

ATG GCG TCC TCC ACG ATG GCC CTC TCC TCC ACC GCC TTC GCC
M A S S T M A L S S T A F A
CCC AAG GCC GTG AAC GTG CCGTCG TCG TCC CCC TTC GAG G R A V N V P S S S A F E
CCC CCC GTG ACC ATG AGO AAG ACGCCC CCG AAG GCC AAG
A R V T * M R K T A A K A K K
CCA CCTCCC CCC TCCGGG AGC CCC TGG TAC GCC CCC ATG
P A A A S G S P W Y G P M
B. PHP8087 - CPN60::GFPm
The mitochondrial signal sequence from the maize chaperonin 60 gene is shown below.

Close, P.S., Master's Theses, Iowa State University(1993). The signal sequence includes intron 1 of cpn60II (marked by -) and includes 2 amino acids from the cpn60II protein beyond the processing site (marked by *)
ATG TAC CCCCCC GCCGCT AGC CTC GCC TCC AAGCCC CGG CAA GCC CGC ACCM Y R A A A
S L A S K A R Q A G S
AGC TCC GCCGCT CCC CAG-gtgagagcagactcgtgtttatacgcgtgtgatgggtctgatg
S S A A R Q gaggatcccgccctcagatttggtaatgttggcctggggttctagtagttctgcgtgcagggctgtg
ggtttgctgcatggtgctgtttattttggtggcgatctgccggaatctgtagttcgctcgcgcaaaa
tctaagctagctcgctaatggcgtactggcgtggggtttcacattaatctacggtggtgaactcgtc
actaccgtcctccagttagctgttagacaccgaatacactgattggcagttgagaaacattgatctg
atccagcagaaatcgatgtcttgtgaaattcgttattttattgtcgtgtaaccttggggcatggcag
tctctaattgatcacgcactcacctctgttgtgtgtgatgctttatag-GTTGGA AGC AGC CTT
V G S R L
GCC TGG AGCAGG AAC TAT CCT CCC ATG
A W S R N Y A A M

C. PHP8144 - Ubi::BAA-GFPm - Cloned sequence includes 1 amino acid beyond processing site (marked by *, lower case bases show linker sequence).

The barley alpha amylase 1 signal sequence is shown below. Knox, C., et al., "Structure and Organization of Two Divergent
Alpha-Amylase Genes From Barley", Plant Mol.

Biol. 9:3-17 (1987). The cloned sequence includes sequence only up to processing site.

ATG GCC AAC AAG CAC CTG AGC CTC TCC CTC TTC CTCCTC CTC CTC CGC CTC TCC
M A N K H L S L S L F L V L L G L S
CCC TCC CTC CCC TCCGGA tcc ATG
A S L A SG S M
D. PHP8757 - Ubi::BAA-GFPm-BLVT - Signal sequence starts at *, lower case bases show linker sequence.

The barley lectin vacuole signal sequence is shown below. Lerner et al., Plant Physiol.

91: 124-129 (1989).

AAG atc tacCTC TTC GCC GAG CCC ATC GCC GCC AAC TCC ACC CTC GTC GCC GAG
K I YV F A E A I A A N S T L V A E
E. PHP8758 - Ubi::BAA-GFPm-HDEL - Signal sequence starts at *, lower case bases show linker sequence.

The HDEL endoplasmic reticulum retention signal from maize b-70 is shown below. Fontes et al., Plant Cell 3: 483-496 (1991).

AAG atctac GAC GGC GGC GTG GAC GAC GAC CAC GAC GAG CTCK I Y *D G G V D D D H D E L
F. PHP8759 - Ubi::BAA-SVT-GFPm - Marked by ^, lower case bases show linker sequence.

The sweet potato sporamin vacuole signal sequence is shown below. Matsuoka et al.,
Proc. Natl. Acad. Sci.8Q : 834 (1991). The sequence shown includes 4 additional sporamin amino acids beyond the processing site (marked by *).

ATG GCC AAC AAG CAC CTG AGC CTC TCC CTC TTC CTC GTG CTC CTC GGC CTC TCC
M A N K H L S L S L F L V L L G L S
GCC TCC CTC GCC TCC GGA CAC AGC AGG TTC AAC CCC ATC AGG G CCC ACC ACC
A S L A S G H S R F N P I R L P T T
CAC GAG CCC GCC AGC AGC GAG ACC ggatac ATG
H E P A S S E T G S M
G. PHP8882 - Ubi::GFPm-SKL - (marked by *)
The consensus peroxisome signal sequence designated SLK is shown below. Gould et al.,
J. Cell Biol 108: 1657 (1989).

AAG TCG AAG CTT
KS K L
H. Ubi::GR-GFPm
The pea glutathione reductase mitochondrial and chloroplast signal sequence is shown below. Creissen et al., Plant J. 2: 129 (1991). The sequence shown includes 20 additional amino acids beyond the putative processing site (marked by*).

ATG AAC CAG GCG ATG GCC ACC CCG CTG TCC CTG TCC TGC TGC TCC CCG ACC CTG
M N Q A M A T P L S L S C C S P T L
ACCAGG TCC ACC CTG TTC TTC ACC AAG ACC TTC CCG TTC TCC CGC TCC TTC TCCT R s T L
F F T K T F P F S R S F S
ACC CCG CTG CCG CTG TCC ACC AAG ACC CTG ATC TCC CTG TCC CCG CCG CAC AGGT P L P
L S T K T L I S L S P P H a
ACC TTC GCC GTG AGG GCT GAG TCC CAG AAC GGC GCG GAC CCG GCC AGG CAG TACE S Q
N G A D P A R Q Y D F D L F S
GAC TTC GAC CTG TTC ACC ATC GGC
I G T F A V*R A

I. PHP9053 - Ubi::NLS-GFPm-MALS
The nuclear localization signal from simian virus 40(SV40) was fused to the N terminus of GFPm.

In order to retain the protein in the nucleus the molecular weight of NLS-GFPm was increased by making
a carboxy terminal addition of the large unrelated protein maize acetolactate synthase (ALS) (lower case
bases show linker sequence).Kalderon,D.

Robers, B., Richardson, W., and Smith A., "A short amino acid sequence able to specify nuclear
location", Cell 39: 499-509 (1984).

CCG CCC AAG AAG AAG CGC AAG GTGcca ATGP P K K K R K V P M
AAG atc cac ATG
K I H M
J. Ubi::GFPm-HRGP
A carboxy terminal fusion of a portion of the maize HRGP coding sequence to GFPm was used to anchor
GFP to the cell wall. Sequence encoding amino acids 177 to 328 was used. Stiefel, V., Ruiz
Avila, L., Raz R., Valles M., Gomez J., Pages M.,
Martinez-Izquierdo J., Ludevid M., Landale J.,
Nelson T., and Puigdomenech P., "Expression of a maize cell wall hydroxyproline-rich glycoprotein gene
in early leaf and root vascular differentiation", Plant Celli: 785-793 (1990).

TAC ACT CCA AGC CCT AAG CCA CCG GCT ACC AAG CCT CCC ACGY T P S P K P P A T K P P T
CCC AAG CCG ACC CCC CCA ACG TAC ACC CCT TCC CCA AAG CCT
P K P T P P T Y T P S P K P
CCG ACA CCC AAG CCG ACC CCC CCC ACG TAC ACC CCT TCT CCC
P T P K P T P P T Y T P S P
AAG CCT CCC ACG CCC AAG CCC ACC CCC CCC ACC TAC ACT CCA
K P P T P K P T P P T Y T P
AGC CCC AAG CCT CCC ACA CAC CCC ACC CCC AAG CCC ACC CCA
S P K P P T H P T P K P T P
CCC ACG TAC ACC CCT TCC CCA AAG CCT CCG ACG CCC AAG CCCP T Y T P S P K P pT P K P
ACC CCA CCC ACG TAC ACC CCT TCC CCA AAG CCT CCC ACA CCC
T P P T Y T P S P K P P T P
AAG CCC ACC CCA CCC ACG TAC ACC CCT TCC CCA AAG CCT CCC
K P T P P T Y T P S P K P P
ACA CCC AAG CCC ACC CCA CCC ACC TAC ACT CCC ACA CCC AAG
T P K P T P P T Y T P T P K
CCG CCG GCC ACC AAG CCG CCC ACC TAC ACT CCG ACG CCG CCG
P P A T K P P T Y T P T P P
CTG TCT CAC ACC CCC AGC CCC CCC CCA CCA TAC TAC
V S H T P S P P P P Y Y
K. Ubi::GFPm-SOD
Superoxide dismutase coding sequence fused to
GFPm. Alcendor D., Chapman G., and Beaman B., "Isolation, sequencing and expression of the
superoxide dismutase-encoding gene (sod) of Nocardia asteroides strain GUH-2", Gene 164: 143-147
(1995).

CTC CCT GAG TAC ACC CTC CCC GAT CTC GAT TAC GAC TAC ACC
V A E Y T L P D L D Y D Y S
CCC TCC AAC CCC ACA TCT CCCCCC AGA TCA ACG ACC TGA CAC
A L E P H I S G Q I N E L H
CAT TCC AAG CAC CAC GCC GCC TAC GTC GCC GGT GCC AAC ACG
H S K H H A A Y V A G A N T
GCA CTC GAG AAG CTG GAA GCC GCC CCT GAG CCC CGC CAT CAC
A L E K L E A A R E A G D H
AGC CGC ATC TTC CTG CAC GAG AAG AAC CTC GCG TTC CAC CTC
S A I F L H E K N L A F H LCCC GGA CAC GTC AAC CAC TCC ATC TGG TGG AAC AAC CTG TCC
G G H V N H S I W W K N L S
CCC AAC GGT GGC GAC AAG CCG GTC GGC GAG CTG GCC GCG GCC
P N G G D K P V G E L A A A
ATC GAC CAC CAG TTCCCT TCG TTC GAC AAG TTC CCC GCG CAG
I D D Q F G S F D K F R A Q

TTC ACC GCCCCC GCC AAC CGC CTG CAC CCC TCGCCC TGG CCG
P T A A A N G L Q G S G W A
GTG CTC GGT TAC GAC ACC CTCCCC CAC AAG CTG CTC ACC TTC
V L G Y D T L G Q K L L T F
CAC CTC TAC GAC CAC CAC GCC AACCTC CCC CTGCCC ATC ATC
Q L Y D Q Q A N V P L G I I
CCG CTG CTC CAG TGC GAC ATG TGG GAG CAC GCC TTC TAC CTG
P L L Q V D M W E H A F Y L
CAG TAC AAG AAC GTC AAG GCC GAC TAC GTG ACC GCG TTC TGG
Q Y K N V K A D Y V T A F W
AAC GTC GTC AAC TGG GCC GAC GTG CAG GAC CGC TTC GGC AAG N v V N W A D V Q D R F G
K
GCC GTC AAC CAG GGCMG GGC CTT ATC TTC GaG A V N Q G K G L I F G 9. Detection of GFP in
Transformed Plant Cells
GFP is detected in transformed plant cells by conventional spectrophometric methods. The transformed
cells or tissue are screened for the presence of GFP protein by means of the illuminating the cells with
UV to blue light and screening for the presence of green fluorescence.

Compound and dissecting microscopes were fitted with appropriate filter combinations for fluorescent
protein excitation. Illumination with W-blue light (exciting around the absorption maximum of 395 nm or
around the minor peak at approximately 475 nm for GFP; around 480490 nm for the red-shifted version
and around 380 nm for the blue fluorescent protein) is required for visualization. A hand-held lamp for
benchtop work also permits good visualization. Cut-off filters or bandpass filters between the fluorescing
tissue and the viewer (i.e. around the intermediate objective or the eyepieces of the microscope, or hand-
held in front of the eyes if working on the benchtop) greatly reduced background autofluorescence from
the tissue. For GFP and the redshifted GFP this cut-off or bandpass filter permitted light between 500-
550 nm to reach the viewer. For the blue fluorescent protein, this filter permitted the passage of 420 to
450 nm light. Useful filters and wavelengths are not restricted to those described above, and further
generic description of the optical characteristics of this system are available. Heim et al., Currently
Biology 6: 178-182 (1996).

10. Foreign Protein Genes and Agronomic Genes
With transgenic plants according to the present invention, a foreign protein can be produced in
commercial quantities. Thus, the selection and propagation techniques described above yield a plurality
of transgenic plants which are harvested in a conventional manner, and a foreign protein then is
extracted from a tissue of interest or from total biomass. Protein extraction from plant biomass can be
accomplished by known methods which are discussed, for example, by Heney and Orr, Anal. Biochem.
114: 92-6 (1981).

According to a preferred embodiment, the transgenic plant provided for commercial production of foreign
protein is maize. In another preferred embodiment, the biomass of interest is seed. For the relatively
small number of transgenic plants that show higher levels of expression, a genetic map can be
generated, primarily via conventional RFLP and PCR analysis, which identifies the approximate
chromosomal location of the integrated DNA molecule. For exemplary methodologies in this regard, see
Glick and Thompson, METHODS IN PLANT MOLECULAR
BIOLOGY AND BIOTECHNOLOGY 259-284 (CRC Press, 1993). Map information concerning
chromosomal location is useful for proprietary protection of a subject transgenic plant. If unauthorized
propagation is undertaken and crosses made with other germplasm, the map of the integration region
can be compared to similar maps for suspect plants, to determine if the latter have a common parentage
with the subject plant. Map comparisons would involve hybridizations, RFLP, PCR and sequencing, all of
which are conventional techniques.

Likewise, by means of the present invention, agronomic genes can be expressed in transformed plants.

More particularly, plants can be genetically engineered to express various phenotypes of agronomic
interest. The genes implicated in this regard include, but are not limited to, those categorized below.

1. Genes That Confer Resistance To Pests or Disease
And That Encode:
(A) Plant disease resistance genes. Plant defenses are often activated by specific interaction between
the product of a disease resistance gene (R) in the plant and the product of a corresponding avirulence

(Avr) gene in the pathogen. A plant variety can be transformed with cloned resistance gene to engineer plants that are resistant to specific pathogen strains.

See, for example Jones et al., Science 266: 789 (1994) (cloning of the tomato Cf-9 gene for resistance to Cladosporium fulvum); Martin et al., Science 262: 1432 (1993) (tomato Pto gene for resistance toPseudomonas syringae pv. tomato encodes a protein kinase); Mindrinos et al., Cell 78: 1089 (1994) (Arabidopsis RSP2 gene for resistance toPseudoinonas syringae).

(B) A Bacillus thuringiensis protein, a derivative thereof or a synthetic polypeptide modeled thereon. See, for example, Geiser et al., Gene 48: 109 (1986), who disclose the cloning and nucleotide sequence of a Bt 6endotoxin gene. Moreover, DNA molecules encoding 6endotoxin genes can be purchased from American Type
Culture Collection (Rockville, MD), under ATCC accession
Nos. 40098, 67136, 31995 and 31998.

(C) A lectin. See, for example, the disclosure by Van Damme et al., Plant Molec. Biol. 24: 825 (1994), who disclose the nucleotide sequences of severalCli via miniata mannose-binding lectin genes.

(D) A vitamin-binding protein such as avidin. See
U.S. patent application serial No. 07/911,864, the contents of which are hereby incorporated by reference.

The application teaches the use of avidin and avidin homologues as larvicides against insect pests.

(E) An enzyme inhibitor, for example, a protease inhibitor or an amylase inhibitor. See, for example, Abe et al., J. Biol.Chem. 262: 16793 (1987) (nucleotide sequence of rice cysteine proteinase inhibitor), Huub et al., Plant.Molec. Biol. 21: 9S5 (1993) (nucleotide sequence of cDNA encoding tobacco proteinase inhibitor
I), and Sumitani et al., Biosci. Biotech. Biochem. 57: 1243 (1993) (nucleotide sequence of Streptomyces nitrosporeusamylase inhibitor).

(F) An insect-specific hormone or pheromone such as an ecdysteroid and juvenile hormone, a variant thereof, a mimetic based thereon, or an antagonist or agonist thereof. See, for example, the disclosure by
Hammock et al., Nature 344: 458 (1990), of baculovirus expression of cloned juvenile hormone esterase, an inactivator of juvenile hormone.

(G) An insect-specific peptide or neuropeptide which, upon expression, disrupts the physiology of the affected pest. For example, see the disclosures of
Regan, J. Biol. Chem. 269: 9 (1994) (expression cloning yields DNA coding for insect diuretic hormone receptor), and Pratt et al., Biochem. Biophys. Res. Comm. 163: 1243 (1989) (an allostatin is identified in Diploptera puntata). See also U.S. patent No. 5,266,317 to Tomalski et al., who disclose genes encoding insect-specific, paralytic neurotoxins.

(H) An insect-specific venom produced in nature by a snake, a wasp, etc. For example, see Pang et al., Gene 116: 165 (1992), for disclosure of heterologous expression in plants of a gene coding for a scorpion insectotoxic peptide.

(I) An enzyme responsible for an hyperaccumulation of a monterpene, a sesquiterpene, a steroid, hydroxamic acid, a phenylpropanoid derivative or another non-protein molecule with insecticidal activity.

(J) An enzyme involved in the modification, including the post-translational modification, of a biologically active molecule; for example, a glycolytic enzyme, a proteolytic enzyme, a lipolytic enzyme, a nuclease, a cyclase, a transaminase, an esterase, a hydrolase, a phosphatase, a kinase, a phosphorylase, a polymerase, an elastase, a chitinase and a glucanase, whether natural or synthetic. See PCT application WO 93/02197 in the name of Scott et al., which discloses the nucleotide sequence of a callase gene. DNA molecules which contain chitinase-encoding sequences can be obtained, for example, from the ATCC under accession Nos.

39637 and 67152. See also Kramer et al., Insect Biochem.

Molec. Biol. 23: 691 (1993), who teach the nucleotide sequence of a cDNA encoding tobacco hookworm

chitinase, and Kawalleck et al., Plant Molec. Biol. 21: 673 (1993), who provide the nucleotide sequence of the parsley ubi4-2 polyubiquitin gene.

(K) A molecule that stimulates signal transduction. For example, see the disclosure by Botella et al., Plant Molec. Biol. 24: 757 (1994), of nucleotide sequences for mung bean calmodulin cDNA clones, and Griess et al., Plant Physiol. 104: 1467 (1994), who provide the nucleotide sequence of a maize calmodulin cDNA clone.

(L) A hydrophobic moment peptide. See U.S. patent applications serial No. 08/168,809 (disclosure of peptide derivatives of Tachyplesin which inhibit fungal plant pathogens) and serial No. 08/179,632 (teaches synthetic antimicrobial peptides that confer disease resistance) the respective contents of which are hereby incorporated by reference.

(M) A membrane permease, a channel former or a channel blocker. For example, see the disclosure by Jaynes et al., Plant Sci. 89: 43 (1993), of heterologous expression of a cecropin-P lytic peptide analog to render transgenic tobacco plants resistant toPseudomonas solanacearum.

(N) A viral-invasive protein or a complex toxin derived therefrom. For example, the accumulation of viral coat proteins in transformed plant cells imparts resistance to viral infection and/or disease development effected by the virus from which the coat protein gene is derived, as well as by related viruses. See Beachy et al., Ann. Rev. Phytopathol. 28: 451 (1990). Coat protein-mediated resistance has been conferred upon transformed plants against alfalfa mosaic virus, cucumber mosaic virus, tobacco streak virus, potato virus X, potato virus Y, tobacco etch virus, tobacco rattle virus and tobacco mosaic virus. Id.

(O) An insect-specific antibody or an immunotoxin derived therefrom. Thus, an antibody targeted to a critical metabolic function in the insect gut would inactivate an affected enzyme, killing the insect. Cf.

Taylor et al., Abstract#497, SEVENTH INT'L SYMPOSIUM ON MOLECULAR.PLANT-MICROBE INTERACTIONS (1994) (enzymatic inactivation in transgenic tobacco via production of single-chain antibody fragments).

(P) A virus-specific antibody. See, for example, Tavladoraki et al., Nature 366: 469 (1993), who show that transgenic plants expressing recombinant antibody genes are protected from virus attack.

(Q) A developmental-arrestive protein produced in nature by a pathogen or a parasite. Thus, fungal endoot- 1,4-D-polygalacturonases facilitate fungal colonization and plant nutrient release by solubilizing plant cell wallhomo-a-I,4-D-galacturonase. See Lamb et al., Bio/Technology10: 1436 (1992). The cloning and characterization of a gene which encodes a bean endopolygalacturonase-inhibiting protein is described by Toubart et al., Plant J. 2: 367 (1992).

(R) A developmental-arrestive protein produced in nature by a plant. For example, Logemann et al., Bio/Technology 10: 305 (1992), have shown that transgenic plants expressing the barley ribosome-inactivating gene have an increased resistance to fungal disease.

2. Genes That Confer Resistance To A Herbicide, For Example:
(A) A herbicide that inhibits the growing point or meristem, such as an imidazalinone or a sulfonylurea.

Exemplary genes in this category code for mutant ALS and AHAS enzyme as described, for example, by Lee et al., EMBO J.?: 1241 (1988), and Miki et al., Theor. Appl.

Genet. 80: 449 (1990), respectively.

(B) Glyphosate (resistance imparted by mutant EPSP synthase and aroA genes, respectively) and other phosphono compounds such as glufosinate (PAT and bar genes), and pyridinoxy or phenoxy proprionic acids and cycloshexones (ACCase inhibitor-encoding genes). See, for example, U.S. patent No. 4,940,835 to Shah et al., which discloses the nucleotide sequence of a form of EPSP which can confer glyphosate resistance. A DNA molecule encoding a mutant aroA gene can be obtained under ATCC accession No. 39256. and the nucleotide sequence of the mutant gene is disclosed in U.S. patent No.

4,769,061 to
Comai. European patent application No. 0 333 033 to
Kumada et al. and U.S. patent No. 4,975,374 to Goodman et al. disclose nucleotide sequences of
glutamine synthetase genes which confer resistance to herbicides such as Lphosphinothricin. The
nucleotide sequence of a phosphinothricin-acetyl-transferase gene is provided in
European application No. 0 242 246 to Leemans et al. De
Greef et al.,Bio/Technology 7: 61 (1989), describe the production of transgenic plants that express
chimeric bar genes coding for phosphinothricin acetyl transferase activity. Exemplary of genes conferring
resistance to phenoxy proprionic acids and cyclohexones, such as sethoxydim and haloxyfop, are
theAccl-Sl, Accl-S2 and
Accl-S3 genes described by Marshall et al., Theor. Appl.

Genet.83: 435 (1992).

(C) A herbicide that inhibits photosynthesis, such as a triazine (psbA and gs+ genes) and a benzonitrile
(nitrilase gene). Przibilla et al., Plant Cell 3: 169 (1991), describe the transformation of Chlamydomonas
with plasmids encoding mutant psbA genes. Nucleotide sequences for nitrilase genes are disclosed in
U.S.

patent No. 4,810,648 to Stalker, and DNA molecules containing these genes are available under ATCC
accession
Nos. 53435, 67441 and 67442. Cloning and expression of
DNA coding for a glutathione S-transferase is described by Hayes et al., Biochem. J. 285: 173 (1992).

3. Genes That Confer Or Contribute To A Value
Added Trait. Such As:
(A) Modified fatty acid metabolism, for example, by transforming a plant with an antisense gene of
stearoyl-ACP desaturase to increase stearic acid content of the plant. See Knultzon et al., Proc. Nat'l
Acad Sci.

USA89: 2624 (1992)
(B) Decreased phytate content.
(1) Introduction of a phytase-encoding gene
would enhance breakdown of phytate, adding more
free phosphate to the transformed plant. For
example, see Van Hartingsveldt et al., Gene 127:
37 (1993), for a disclosure of the nucleotide
sequence of an Aspergillus niger phytase gene.

(2) A gene could be introduced that reduces
phytate content. In maize, this, for example,
could be accomplished, by cloning and then re
introducing DNA associated with the single allele
which is responsible for maize mutants
characterized by low levels of phytic acid. See
Raboy et al., Maydica 35: 383 (1990).

(C) Modified carbohydrate composition effected, for example, by transforming plants with a gene coding
for an enzyme that alters the branching pattern of starch. See Shiroza et al., J. Bacterial. 170: 810 (1988)
(nucleotide sequence of Streptococcusmutans fructosyltransferase gene), Steinmetz et al., Mol. Gen.

Genet. 200: 220 (1985) (nucleotide sequence of Bacillus subtilis levansucrase gene), Pen et al.,
Bio/Technology 10: 292 (1992) (production of transgenic plants that express Bacillus licheniformis a-
amylase), Elliot et al.,
Plant Molec. Biol. 21: 515 (1993) (nucleotide sequences of tomato invertase genes),Sgaard et al., J. Biol.

Chem. 268: 22480 (1993) (site-directed mutagenesis of barley amylase gene), and Fisher et al., Plant
Physiol.

102: 1045 (1993) (maize endosperm starch branching enzyme
II).

Example 1
Construction of Genes and Vectors
The GFPm nucleotide sequence was derived from a back translation of the protein sequence using maize preferred codons. Sequence analysis was performed using the Wisconsin Sequence Analysis Package from Genetics
Computer Group, Madison, WI. The nucleotide sequence was assembled from a series of synthetic oligonucleotides.

Cloning sites include a 5-prime flankingBamHI restriction site, an AflIII site at the start codon, a 3prime flanking HpaI site or a BglII site converting the stop codon to an isoleucine (Fig. 1).

Amino terminal fusions to GFPm were created using synthetic oligonucleotides encoding the signal sequence flanked by a 5-primeBamHI orEgill restriction site and 3-prime NcoI site. The synthetic signal sequences were cloned intoPHP7921 (Fig. 4) using the 5-prime flanking
BamHI and AflIII restriction sites of GFPm to create inframe fusions. Sequences cloned in this manner include the maize chloroplast signal sequence, barley alphaamylase signal sequence, pea glutathione reductase signal sequence, and SV40 nuclear localization signal. The sporamin vacuole signal sequence was synthesized with a 5-prime BspEI and 3-prime BamHI for insertion between the
BAA pre-sequence and GFPm coding sequence of pHP8144 (Fig. 5). TheCPN60 promoter site, created by sitedirected mutagenesis, was used with the GFPm AflIII site to make the fusion sequence. Each of plasmids PHP7921 andPHP8144 are based onpUC18.

Carboxy terminal sequences were synthesized with a 5-prime BglII and 3-prime HpaI site, and cloned into pHP7921 or pHP8144 using the 3-prime BflII and HpaI sites of GFPm. The barley lectin vacuole target, HDEL ER retention signal, SKL peroxisome signal, ALS coding sequence and HRGP coding sequence fusions were made using this strategy.

TABLE 1
PLASMIDS
EMI51.1.

Targeting
<tb> <SEP> PHP <SEP> Target <SEP> Promoter <SEP> 5' <SEP> 3' <SEP> Localized <SEP> Other
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Gene <SEP> Sequence
<tb> 7956 <SEP> maize <SEP> 4xERE <SEP> -- <SEP> -- <SEP> Cytoplasm <SEP> Omega <SEP>
GFPm <SEP> pinII <SEP> Estrogen
<SEP> Prime <SEP> (O') <SEP> inducible
<tb> Adh1 <SEP> expression
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EMI52.1

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GFPm
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GFPm <SEP> pinII <SEP> Sweet <SEP> potato
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EMI53.1

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EMI54.1

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<tb> 8674 <SEP> maize <SEP> 2x35s <SEP> -- <SEP> -- <SEP> cytoplasm <SEP> O' <SEP> Adhl
<SEP> FRT-bar- <SEP> pinII <SEP> Select <SEP> on
<tb> intron <SEP> FRT-GFPm <SEP> bialaphos,
<tb> then <SEP> excise
<tb> bar <SEP> and
<tb> activate <SEP> GFP
<tb> 8911 <SEP> maize <SEP> ubiquitin <SEP> -- <SEP> adhl <SEP> cytoplasm <SEP> Ds <SEP>
GFPm <SEP> pinII <SEP> ubi-Ds-2x35S:
<tb> /2x35s.<SEP> intron <SEP> I <SEP> bar <SEP> pinII <SEP> :bar::pin-Ds
GFPm::pinII
<tb> 8088 <SEP> maize <SEP> ubiquitin <SEP> -- <SEP> -- <SEP> cytoplasm <SEP> -- <SEP>
GFPm-S65T <SEP> pinII <SEP> Red-shifted
<tb> GFPm
<tb> 8138 <SEP> maize <SEP> ubiquitin <SEP> -- <SEP> -- <SEP> cytoplasm <SEP> -- <SEP>
GFPm-Red2 <SEP> pinII <SEP> Red-Shifted
<tb> version <SEP> #2
<tb> F64M, <SEP> S65G,
<tb> Q69L
<tb>
EMI55.1

8175 <SEP> maize <SEP> ubiquitin <SEP> -- <SEP> -- <SEP> cytoplasm <SEP> -- <SEP> Blue
<SEP> FP <SEP> pinII <SEP> GFPm-modified
<tb> Y66H
<tb> 8007 <SEP> maize <SEP> 2x35s <SEP> adh1 <SEP> pinII <SEP> Tandem <SEP> vector
<tb> ubiquitin <SEP> intronl::bar <SEP> pinII <SEP> for <SEP> FLP
<tb> ubi <SEP> excision <SEP> of
<tb> intron::FLP <SEP> FRT <SEP> to
<tb> activate <SEP> GFPm
<tb> 8011 <SEP> sunflo <SEP> 2x35s <SEP> -- <SEP> -- <SEP> cytoplasm <SEP> O' <SEP> GFPm
<SEP> pinII <SEP> Binary
<tb> wer <SEP> ALS <SEP> -- <SEP> -- <SEP> NPT-II <SEP> pinII
<tb> 8491 <SEP> sunflo <SEP> 2x35s <SEP> -- <SEP> -- <SEP> cytoplasm <SEP> O' <SEP> GFPm-
S65T <SEP> pinII <SEP> Binary <SEP> with
<tb> wer <SEP> ALS <SEP> -- <SEP> -- <SEP> NPT-II <SEP> pinII <SEP> red-shifted
<tb> GFP
<tb>
The ubiquitin promoter is described in
Christensen et al., Plant Mol. Biol. 18: 675-689 (1992). The 4xERE promoter is described in Klein
Hitpass et al., Cell 46: 1053-1061 (1986). The CPN60; 2x35S and ALS promoters are described in Close

P. Ph.D.

Dissertation, Iowa State University; Gardner et al.,
Nucl. Acid Res. 9: 2871-2888 (1981) and PHI Patent
Application 08/409,297, respectively. The Omega prime(O') 5-prime sequence is described by Gallie et al.,
Nucl. Acids Res. 15: 3257-3273 (1987) while the Adh 1 intron I is described by Dennis et al., Nucl. Acids Res. 12: 3983-3990 (1984). The 3-prime sequence pinII is described in An et al., Plant Cell 1: 115-122 (1989). Structural genes used in the construction of the vectors include the GFP gene (native jellyfish) described by Prasher et al., Gene 111: 229-233 (1992); the maize ALS gene described by Fromm et al., Bio/Technol. 8: 883 (1990) and the maize HPRG described by Steifel et al., Plant Mol. Biol. 11: 483-493 (1988).

The FLP/FRT system contained in plasmidsPHP8674 andPHP8007 is described in O'Gorman et al., Science251: 1351-1355 (1991) and Jayaram M., Proc. Natl. Acad. Sci.

USA 82: 5875-5879 (1985). The Ac/Ds system is described by Muller-Neumann et al., Mol. Gen. Gent.

198: 19-24(1984)
ForAgrobacter um-mediated transformation of sunflower, the backbone of PHP8011 is a bin 19-based vector. Beven,Nucl. Acids Res. 12: 8711-8721 (1984).

The NPT-II cassette was removed from bin 19 and the following cloned between the left and right border sequences; the double35S promoter; omega', the GFPm coding sequence and the pinII 3' region (all described above) were cloned downstream of the left border sequence, followed in a head-to-tail orientation by theBnALS3 promoter; the NPT-II coding sequence and the pinII 3' region. Beven, "Binary Agrobacterium Vectors for Plant Transformation", Nucl. Acids Res. 12: 87118721(1984).

GFPr, BFP and GFPs (soluble GFP) were constructed by means of oligonucleotide-directed mutagenesis of the GFPm nucleotide sequence shown in
Figure 1 as described by Ausubel, supra. With regard to GFPr, TCC was changed to ACG resulting in the S65T substitution in the GFP amino acid sequence. With regard to BFP, TAC was changed to CAC, resulting in the
Y66H substitution in the GFP amino acid sequence.

With regard to GFPs, the following sequence changes in GFPm were made. TCC was changed to AGC, resulting in aF99S substitution in the GFP amino acid sequence. The oligonucleotide used to introduce this mutation also caused a silent mutation at R96 (AGC changed to CGG), thereby adding a BsrBI restriction site. ATG was changed to ACC, resulting in an M153T substitution in the GFP amino acid sequence. GTG was changed to GCC, resulting in a V163A substitution in the GFP amino acid sequence. A single oligonucleotide was used to change the amino acids at 153 and 163.

This oligonucleotide also caused a silent mutation at
A154 (GCC changed to GCG), which added a SacII restriction site. As reported by Crameri et al., supra, these three amino acid changes did not alter the absorption/emission characteristics of GFP. These three amino acid substitutions, however, increased the solubility of GFP. Maize cells transformed with the GFPs gene fluoresce in blue to UV light based on transient assays.

Example 2
MaizeTrknsfolrmation with GFP Screening or Bialaphos
Selection
MaizeHi-II immature embryos were co-transformed via microprojectile bombardment with a mixture of PHP7814 (ubi::PAT::pinII) and PHP8409. Plasmid PHP7814 carries the PAT gene encoding resistance to bialaphos operably linked to the ubiquitin promoter and the terminator from the pinII gene. Standard CaCl2/spermidine precipiation of DNA onto tungsten microprojectiles was used to prepare the DNA-particles.

Prior to bombardment, the embryos of approximately 1.52.0 mm in length were cultured on 560P medium including
N6 salts, Erikkson's vitamins, 0.69 mg/L proline, 2 mg/L 2,4-D, and 3% sucrose. After 4-5 days of incubation in the dark at 280C, embryos were removed from 560P medium and cultured, coleorhizal end up, on 560L medium which is equivalent to 560P except that it contains 12% sucrose. Embryos were

allowed to acclimate to this medium for 3 h prior to transformation.

Embryos were transformed using the PDS-1000
Helium Gun from Bio-Rad at one shot per sample using 650 PSI rupture disks. On average approximately 0.0067μg of DNA was delivered per shot. For each treatment (pat gene with bialaphos selection or GFP gene with screening for GFP), 447 embryos were used. For the bialaphos-selection treatment; following bombardment, the embryos were maintained on 560L medium for 48 h before transfer to 560R selection medium containing N6 salts, Eriksson's vitamins, 2 mg/L 2,4-D, 3% sucrose and 3mg/L bialaphos. Plates were maintained at 28OC in the dark and were observed for colony recovery.

Transfer of colonies to fresh medium occurred every two weeks. Transgenic colony recovery under bialaphos selection was accomplished by picking the healthy, growing transgenic calli from amongst the inhibited background of non-transgenic tissue as the material was progressively transferred to fresh media. For the GFP screening treatment, 560P media was used after bombardment and the two-day recovery on 560L. Calli were transferred to fresh non-selective 560P medium every two weeks. Screening for GFP expression was carried out at each transfer using a Xenon and/or
Mercury light source with the appropriate filters for
GFP visualization.

Table 2
Recovery of Transgenic Events in MaizeHi-II Callus
Using
Either GFP Visualization or Bialaphos Selection
EMI59.1

	Number of Events Recovered	
Selection Method	Initial Events	Events Capable of Regeneration
Bialaphos selection	16	12
Screened using GFP	9	3

Transgenic events were recovered from each treatment. The largest number of transgenic events were consistently obtained by means of bialaphos selection. However, the frequency of recovery of transgenic events with GFP screening was approximately one-half the rate of recovery with bialaphos selection which was surprisingly high considering that GFP screening does not rely on selection of transgenic events in the presence of a cellular toxin.

Once GFP expressing colonies were identified they were monitored regularly for new growth and expression using the Xenon light source. Plant cells containing GFP were regenerated by transferring the callus to 288 medium containing MS salts, 1 mg/L IAA, 0.5 mg/L zeatin and 4% sucrose. The callus was placed in the light. As plantlets developed they were transferred to tubes containing 272K, hormone-free MS medium and 3% sucrose. The percentage of green fluorescent colonies that regenerated into whole plants was determined.

Leaf samples were collected from TO transgenic plants from events recovered by means of bialaphos selection, or for events recovered by means of GFP visualization (non-selected). Genomic DNA was extracted from all leaf samples and Southern analysis was performed using the PAT and GFP genes as hybridization probes. Hybridization for the PAT gene and GFP gene were performed sequentially on the same blots, by stripping and reprobing for the other transgene. Bands hybridizing for the PAT gene and for the GFP gene were observed in all 3 GFP-screened events tested and in all 12 of the bialaphos selected events tested. Copy numbers and integration patterns for both treatments appeared similar, and were consistent with previous experiments by the inventors using bialaphos selection of callus. Copy numbers ranged from low (single or two copies for each gene) to numerous (i.e., > 5 copies pergene). Integration events were predominantly at single integration sites but in some cases integration at multiple locations in the genome was observed.

Bialaphos is the most efficient chemical selective agent for maize. Consequently, the high frequency with which transgenic events were obtained with GFP screening, compared to bialaphos selection, was

unexpected. An advantage of GFP screening over bialaphos selection is that non-transformed tissue is not killed. Accordingly, transgenic sectors containing the GFP gene develop in a healthier environment for growth and development.

Example 3
The GFP Marker in Maize Cytoplasm, Chloroplast and
Mitochondria
Maize A188 x B73-derived cells were transformed with plasmidsPHP8080, PHP7921 andPHP8087. Each of these plasmids is described in Example 1 and Table 1.

PlasmidPHP8080 carries the GFPm gene operably linked to the ubiquitin promoter and a chloroplast signal sequence. GFP is directed to the chloroplasts of cells transformed withPHP8080. Plasmid PHP7921 carries the
GFPm gene operably linked to ubiquitin promoter. GFP remains in the cytoplasm of cells transformed withpPGP7921. PlasmidPHP8087 carries the GFPm gene operably lined to theCPN60 promoter andCPN60 signal sequence. GFP is directed to mitochondria of cells transformed with pPGP8087.

The cells were transformed by culturing maize embryos approximately 1.5 to 2 mm in length onto 560P medium containing N6 salts, Erikkson's vitamins, 0.69 g/L Proline, 2 mg/L 2,4-D and 3% sucrose. After 4-5 days of incubation in the dark at28 C, embryos were removed from 560P medium and cultured, coleorhizal end up, onto 560L medium which is equivalent to 560P but contains 12% sucrose. Embryos were allowed to acclimate to this medium for 3 h prior to transformation. Embryos were transformed using the
PDS-1000 Helium Gun from Bio-Rad at one shot per sample using 650PSI rupture disks. DNA delivered per shot averaged at 0.0667yg. Following bombardment, all embryos were maintained on 560L medium for 48 hours before transfer to38 sucrose and 3 mg/L bialaphos.

Plates were maintained at 280C in the dark and were observed for colony recovery with transfers to fresh medium occurring every two weeks. Transgenic colony recovery was noted initially as growing callus tissue with a healthy phenotype on selection. Screening for
GFP expression was completed with each transfer using a
Xenon light source with W filter attachments.

GFP localization was confirmed by analysis of TO callus. Samples of calli from the targeted GFP transformations were fixed in FAA and then examined using an inverted microscopes with W filters to visualize GFP. For each targeted construct GFP expression was localized to the specified organelle.

Once GFP expressing colonies were identified they were monitored regularly for new growth and expression using the Xenon light source. Plant cells containing GFP were regenerated by transferring the callus to 288 medium containing MS salts, 1 mg/L IAA, 0.5 mg/L zeatin and4k sucrose. The callus was placed in the light. As plantlets developed they were transferred to tubes containing 272K, hormone-free MS medium and 3% sucrose. The percentage of green fluorescent colonies that regenerated into whole plants was determined.

Regeneration of plants from callus transformed withPHP8080, PHP7921, andPHP8087 appeared to vary between constructs; plants were successfully regenerated from independent transformed calli at respective frequencies of35, 54 and 86%.

Transformation was confirmed by Southern blot analyses using both the BAR and GFPm genes as hybridization probes. GFP localization in various subcellular compartments was confirmed in stable transgenic maize cells using epifluorescent microscopy and image enhancement software.

Table 3
Regeneration Frequency Of Maize Transformed With GFP
Directed to Different Subcellular Compartments
EMI63.1

<tb> Chlorolaflt
<tb> <SEP> Exp <SEP> # <SEP> Total <SEP> Green <SEP> Regenerate <SEP> Percentage <SEP> Ave.
<tb>

<SEP> Colonies <SEP> Plant
<tb> <SEP> Event
<tb> <SEP> ..4.01 <SEP> 32 <SEP> 23 <SEP> 4 <SEP> 17 <SEP> 6
<tb> <SEP> .4.03 <SEP> 0
<tb> <SEP> .4.06 <SEP> 13 <SEP> 11 <SEP> 8 ·
<tb> <SEP> Totals <SEP> 45 <SEP> 34 <SEP> 12 <SEP> 35*
<tb> <SEP> Cytopls
<tb> <SEP> Exp <SEP> # <SEP> Total <SEP> Green <SEP> Regenerate <SEP> Percentage <SEP> Ave.
<tb>

<SEP> Colonies <SEP> PlantsE
<tb> <SEP> vent
<tb> <SEP> .4.01 <SEP> 7 <SEP> 6 <SEP> ~ <SEP> 2 <SEP> 33 <SEP> 4
<tb> <SEP> ..4.03 <SEP> 7 <SEP> 5 <SEP> 4 <SEP> 80 <SEP> 5
<tb> <SEP> ..4.06 <SEP> 8 <SEP> 7 <SEP> 6 <SEP> 86 <SEP> r
<tb> <SEP> ..4.08 <SEP> ~9~ <SEP> 9 <SEP> 3 <SEP> 33 <SEP> 2
<tb> <SEP> 3 <SEP> 3 <SEP> 1 <SEP> 33 <SEP> 4
<tb> <SEP> To~tal~s <SEP> ~~~ <SEP> 34 <SEP> 30 <SEP> 16 <SEP> 51
<tb> Nitochondrial
<tb> <SEP> Exp <SEP> # <SEP> Total <SEP> Green <SEP> Regenerate <SEP> Percentage <SEP> Ave
<tb> <SEP> Colonies <SEP> Plants
<tb> <SEP> .. <SEP> 4.03 <SEP> 5 <SEP> ~ <SEP> 3 <SEP> ~ <SEP> 1 <SEP> 33 <SEP> 9 <SEP> 7
<tb> <SEP> .. <SEP> 4.06 <SEP> 13 <SEP> 11 <SEP> 11 <SEP> 1OO <SEP> 4
<tb> <SEP> Totals <SEP> 1B <SEP> 14 <SEP> 12 <SEP> 86*
<tb> * This is not a mean regeneration frequency, but instead is calculated from the totals for all treatments.

Transformed plants containing GFP in the mitochondrial subcellular compartment grew more vigorously than plants containing GFP in the cytoplasm or chloroplast. The transformed plants containing GFP in the mitochondria were equivalent to the controls in terms of growth rate and appearance.

In contrast, transformed plants containing GFP in the chloroplast were difficult to regenerate. This callus produced a preponderance of roots with few shoots. Almost all the plants containing GFP in the chloroplast that were regenerated and reached maturity clearly exhibited stress with narrow and somewhat flaccid leaves, reduced stature, yellow leaves (presumably caused by reduced chlorophyll levels) and male sterility.

Germination of T1 embryos have been tested in the following manner. Aliquots of seed from a number of independent transformants were imbibed and the embryos were excised and placed on hormone-free MS medium with 3% sucrose for germination (this in vitro test made it easier to visualize the seedling). All embryos were germinated in the dark and then scored for visual GFP expression using W-blue excitation under the dissecting microscope.

For each event tested aliquots of 5 or 10 seeds/event were imbibed, excised and scored for germination and fluorescence. The germination frequency for excised progeny produced on plants transformed with PHP7921 and PHP8080 was good. The germination frequency for excised progeny produced on plants transformed with PHP8087 was good for 2 events (10/10), low for one event (2/10). No detectable germination was observed for 2 events. This lowered germination frequency could be due to mitochondrially expressed GFP expression per se, but it could also be the result of event-to-event variation sometimes observed in transgenic lines.

Germination of a single event from plants transformed with PHP7921 was tested differently. Seed were screened using a W-blue light source, and 10 seed with GFP-expressing seed (embryos and endosperm) were selected. Five of these seed were planted in soil, and in the other 5 the embryos were excised and germinated on medium. None of the seed in soil germinated, while all excised embryos germinated, grew and the seedlings continued to fluoresce. For events where germination appears to be negatively impacted, this could be due to increased GFP concentrations during seed maturation and dry-down. High GFP concentrations and aggregation of the protein has been found to be toxic in E. coli.

Crameri et al., Nature Biotechnol. 14: 315-319 (1996).

Table 4
T1 Progeny Analysis
Plant Germination and GFPm Expression
EMI66.1

Plasmid	Event	# of TI Tested Seed Per Event	# of TI Seed Germinated	# of TI Plants Expressing GFPm
PHP8087: CPN60::mit-GFPm-PinII	1	10	0	0
PHP8087: CPN60::mit-GFPm-PinII	2	10	0	0
PHP8087: CPN60::mit-GFPm-PinII	3	10	10	1
PHP8078:: CPN60::mit-GFPm-PinII	4	10	2	0
PHP8087: CPN60::mit-GFPm-PinII	5	10	10	2
PHP7921: Ubi::Ubi intron-GFPm-PinII	1	10	10	4
PHP8080: Ubi::Ubi intron-CTP-GFPm-PinII	1	5	5	0
PHP8080: Ubi::Ubi intron-CTP-GFPm-PinII	2	5	4	0
PHP8080: Ubi::Ubi intron-CTP-GFPm-PinII	3	5	4	0
PHP48IO: Ubi::Ubi intron-BAR-PinII	1	5	5	0
*PHP7921: Ubi::Ubi intron-GFPm-PinII	2	10	5	5

Germination occured only in vitro cultures GFPm+ 5 germinated in soil 5 germinated in vitro planted in soil were Germination also from Seed in planted Germination Seed

* For all seed except those designated in thePHP7921 transformation, embryos were excised from imbibed seed and germinated on MS medium containing no hormones and 3% sucrose. All embryos were germinated in the dark to allow for visualization of GFPm expression. One event from PHP7921 was tested by planting one half of the seed in soil and one half with embryos excised from endosperm with culture in vitro.

All seed planted in soil did not germinate. The germination rate for embryos excised from the endosperm and cultured in vitro was100%. All plants expressed GFPm.

Example 4
Sunflower Transformation with GFP Screening, GUS
Screening and Kanamycin Selection
Experiments were undertaken to test GFP, GUS and NPTII as marker genes for sunflower transformation.

Two different kinds of meristem systems (split meristem; intact meristem) and two different kinds of particle systems (FG, focusing gun; He, helium gun) were used. The screenable markers GFP and GUS were tested with the intact meristem system under conditions involving no chemical selection but rather selection of transformants based on visual (GFP) or assayable (GUS) expression in transformed sectors.

The NPTII marker gene was tested with the split meristem method using kanamycin for chemical selection of transformants and assays for NPTII expression to identify transformed sectors. In some cases, the experiments were carried only so far as identifying transformed sectors while other experiments were carried out all the way through to test for seed transmission of the marker gene to progeny. Transmission to progeny was confirmed by NPTII ELISA.

Regardless of the exact meristem system or type of particle gun used, the basic transformation protocol involved a combination of wounding by particle bombardment, followed by use of Agrobacterium for DNA delivery, as described by Bidney et al., Plant Mol.

Biol. 18: 301-313 (1992). The plasmids used for plant transformation in these experiments werePHP167 containing GUS as the screenable marker, PHP8011 containing GFP as the screenable marker as well as the
NPTII gene, and PHP762 containing NPTII as the selectable marker. BothPHP167 and PHP762 are binary vectors derived frompGA473. See An et al., EMBO J. 4: 277-284 (1985). The binary backbone forPHP8011 is bin 19. See Bevan, NAR 12: 8711-8721(1984).

Procedures for preparation ofAgrobacterium and preparation of particles for all of the experiments are described in Bidney etal., supra. The Pioneer sunflower line SMF3 was used in all experiments. See Burrus et al., Plant Cell Rep.10: 161-166 (1991) for information concerning SMF3. The Agrobacterium strainEHA101 is described in Bidney et al. supra and strainEHA1OS is described in Hood et al., Transgen. Res. 2: 208-218 (1993). Procedures for use of the helium gun, intact meristem preparation, tissue culture and cocultivation conditions, and transgenic plant recovery for the intact meristem experiments are also described in Bidney et al., supra. Procedures for the use of the focusing gun, i.e., as in the intact meristem experiments, are described in Sautter et al.,
Bio/Technol.: 1080-1085 (1991). Procedures for use of the helium gun, split meristem preparation, tissue culture, co-cultivation, kanamycin selection and transgenic plant recovery for the split meristem experiments are described in Malone-Schoneberg et al.,
Plant Sci. 103: 199-207(1994).

Parameters and general use of the focusing gun were similar to those 1.4ijm described in Sautter et al., supra. Gold particles measuring at a concentration of0.5x106/l and 80 bars of nitrogen were typically used although different sized particles (0.25 to2.5cm) and different nitrogen pressures (40160 bar) were tested. For the Dupont PDS-1000 helium driven particle bombardment device, the meristems were bombarded twice at the highest shelf with 600 psi rupture discs and a vacuum of 26 inches Hg and 1.8Sm tungsten particles. The intact meristem transformation method involved imbibing seed for 24 hours in the dark, removing the cotyledons and root radical, followed by culturing of the meristem explants. Twenty-four hours later, the primary leaves were removed to expose the apical meristem. The explants were placed apical dome side up and bombarded twice with particles, followed by co-cultivation with Agrobacterium. To start the cocultivation for intact meristems a0.5S1 droplet ofor600 4.0 Agrobacterium was placed on the meristem.

After a 3-day co-cultivation the meristems were transferred to culture medium with 250Zg/ml cefotaxime (+ 100 mg/l kanamycin for the NPTII selection experiments). Selection has also been done using 50 gm/l kanamycin. The split meristem method involved imbibing seed, breaking of the cotyledons to produce a clean fracture at the plane of the embryonic axis, excising the root tip and then bisecting the explants longitudinally between the primordial leaves. The two halves were placed cut surface up on the medium then bombarded twice with particles, followed by cocultivation with Agrobacterium. For split meristems, after bombardment the meristems were placed in anOD600 0.6 Agrobacterium suspension for 30 minutes. They were then removed from the suspension onto solid culture medium for 3-day co-cultivation. After this period, the meristems were transferred to fresh medium with 250yg/ml cefotaxime (+lOOmg/l kanamycin for selection).

Selection has also been done using 50 gm/l kanamycin.

Table 5 summarizes the results from experiments looking at the frequency of transformed sectors in primary shoots and secondary branches. Table 6 summarizes the results from experiments carried through to progeny for analysis of seed transmission.

Transformants were identified in either seed or plants by assaying for marker gene expression. For GUS expression, activity was determined using either histochemical staining or the fluorometric assay, see

Jefferson et al., EMBO J. 6: 3901-3907 (1987).

Inheritance of transgene expression was assessed using an NPTII ELISA.

TABLE S
Overall Transformed Sector Frequencies
for Sunflower Transformation
EMI70.1

	PLASMID	MERISTEM	AVE.	%	REPS
MARKER	PHP #	GUN	PREPARATION	SECTORS	(N)
Screening:					
GUS	167	FG	Intact	22.0	15
GUS	167	FG	Intact	15.0	3
GUS	167	He	Intact	13.6	12
GFP	9011	He	Intact	7.5	1
Selection:					
NPTII/Kan.	762	FG	Intact	12.7	3

TABLE 6
Overall Sector and Seed Transmission
for Sunflower Transformation
EMI70.2

	PLASMID	MERISTEM	AVE.	%	REPS	SEED
MARKER	PHP #	GUN	PREPAR-ATION	SECTORS (N)		TRANSMISSION
Screening:						
GUS	167	FG	Intact	15.0	3	0/8
GFP	8011	He	Intact	7.5	1	9/10
Selection:						
NPTII/KAN	762	PG	Intact	12.7	4	0/2
NPTII/KAN	= 8011	He	Split	1.9	4	6/8

For evaluation of initial sector frequencies,
GUS seemed to be the most effective marker gene (Tables 5 and 6). However, based on screening alone (no selection) the observed GUS sectors did not subsequently contribute to the germline and thus did not transmit to progeny (Table 6). It was unlikely that this was due to the use of the focusing gun because, both quantitatively and qualitatively, mapping of GUS sectors after use of each type of gun was comparable. GFP was significantly better than NPTII (7.5 vs.1.8k, see Table 6) for both sector frequency and seed transmission when compared using the helium gun. Progeny from GFP-expressing (no chemical selection but instead visually selecting using GFP) TO plants were analyzed for NPTII expression (Table 6)
Progeny from 10 plants were tested and 9 showed inheritance of NPTII activity. From kanamycin-selected
TO plants, 6 out of 8 tested showed transmission of
NPTII expression to the progeny. Not only has GFP "visual selection" worked unexpectedly well for recovering the primary transformants (i.e. compare
Tables 3 and 4), but cotransformation and inheritance of transgene expression was also very efficient.

Table 7: Progeny Analysis of Transformation Experiments ofSMFS
with GFP (PHP8011: CaMV-GFPm,ALS-NPIII)
EMI72.1

Treatment	SID	No.	of	TO	NPTII	Maximum	Transmissi
(Comments)	Seeds	Map	NPTII	on			

<tb> <SEP> Screened <SEP> Range <SEP> Efficiency
<tb> <SEP> 281976 <SEP> 246 <SEP> 12.6– > 30 <SEP> 9.9
<tb> <SEP> 281977 <SEP> 13916.3- > 11.9 <SEP>
<tb> <SEP> 281978 <SEP> 127 <SEP> > 30 <SEP> > 30
<tb> <SEP> 281979 <SEP> 175 <SEP> 9-27 <SEP> 6
<tb> Topdown <SEP> Non-selected <SEP> 4
<tb> <SEP> 281980 <SEP> 74 <SEP> > 30 <SEP> 4
<tb> <SEP> 281983 <SEP> 15 <SEP> 3–21.3 <SEP> 6.5 <SEP> 9/10
<tb> (from <SEP> I <SEP> experiment, <SEP> 319454 <SEP> 124 <SEP> 4.1–18.7 <SEP> 5.9
<tb> 120 <SEP> explants <SEP> in <SEP> total) <SEP>
<tb> <SEP> 319453 <SEP> 118 <SEP> ++ <SEP> 8.6
<tb> <SEP> 281982 <SEP> 47 <SEP> > 30 <SEP> 6.2
<tb> <SEP> 281981 <SEP> 3 <SEP> > 30 <SEP> 0
<tb> <SEP> 248112 <SEP> 6 <SEP> ++ <SEP> 2.3
<tb> <SEP> 248113 <SEP> 24 <SEP> > 30 <SEP> 7.8
<tb> <SEP> 248111 <SEP> 41 <SEP> > 30 <SEP> > 30
<tb> Split <SEP> selected
<tb> <SEP> 248107 <SEP> 23 <SEP> 17 <SEP> 2.7
<tb> <SEP> 248114 <SEP> 67 <SEP> > 30 <SEP> 1.2
<tb> (from <SEP> 4 <SEP> experiments, <SEP> 248110 <SEP> 8 <SEP> 4.1 <SEP> 0 <SEP> 618
<tb>955 <SEP> explants <SEP> in <SEP> total)
<tb> <SEP> 248109 <SEP> 6 <SEP> 10.7 <SEP> 0 <SEP>
<tb> <SEP> 248115 <SEP> 86 <SEP> ++ <SEP> <SEP>
<tb>

Parallel experiments comparing GFP screening, GUS screening and NPTII/kanamycin selection can be performed using intact meristems, the Agrobacterium strain EHA1OS and the helium gun, in side-by-side comparisons. Plasmid PHP8011 can be used for GFP/NPII comparisons. An identical plasmid substituting the GUS structural gene for GFP can be used for GUS/NPTII comparisons, and both plasmids can be used in side-by-side experiments for GUS/GFP comparisons. Sector frequency in primary shoots and secondary shoots can be assayed and calculated as described above. Inheritance and expression of transgenes can also be analyzed in these experiments.

Example 5
GFP As A FLP-Mediated Excision Marker In Maize
Immature embryos of the Hi-II genotype were bombarded with PHP8674 which contains a GFPm reporter gene that is activated upon FLP recombinase-mediated.
FRT excision. Plasmid PHP8674 also carries a constitutively expressed gene for bialaphos resistance.

Stably transformed callus was selected on bialaphos and examined under UV-blue light for GFP expression. No
GFP expression was observed in these primary transformants. Pieces of callus transformed with PHP8674 were then bombarded with PHP8007. Plasmid
PHP8007 contains the FLP recombinase gene operably linked to the ubiquitin promoter. Callus sectors expressing GFP were first observed ten days after the second bombardment. These GFP-expressing sectors continued to grow and express the transgene after one month of culture. As a negative control the FRT/GFPm stably transformed callus was also bombarded with a non-FLP containing plasmid. This treatment did not produce GFP expressing sectors.

As a second control for this experiment the consecutive treatments were- done in opposite order.

Hi-II immature embryos were bombarded with PHP8007 which carries the FLP recombinase gene and a bialaphos resistance gene. Stable transformants were recovered following selection on medium containing bialaphos.

These calli were re-bombarded with PHP8674. GFP expression was observed in the callus but this faded over 5-10 days until it could no longer be detected.

The stably transformed callus expresses FLP recombinase. Introduction of PHP8674 likely provided the substrate for excision (the FRT sites along with spacer DNA between the sites) which activated the GFP cassette while still on the plasmid. This "activated expression" was only transient and disappeared as the plasmid was degraded.

Example 6

GFP Targeting to the Nucleus as a Fusion Protein

PlasmidPHP9053 was constructed in which the GFP protein was fused to the ALS enzyme (designated MALS for maize-ALS). The ALS gene, which encodes a protein normally found in the cytoplasm, is typically used to confer herbicide resistance. Fang et al., Plant Mol.

Biol. 18:1185-1187 (1992). A nuclear localization signal from simian virus 40 was fused to the N terminus of GFPm as described in Example 1 (Kalderon et al.,
Cell 39: 499-509 (1984) with the ALS enzyme fused to the C terminus. The sequence encoding this nuclear targeted fusion protein (in construct PHP9053) was introduced into maize cells using particle bombardment as described previously. Once gene expression was confirmed through GFP fluorescence under the microscope, tissue was fixed as described earlier and examined to determine subcellular localization of GFP.

Fluorescence was localized to the nucleus. Plant regeneration and assessment of toxicity for this experiment has not yet been done.

GFP was targeted to the nucleus by fusing the protein to another polypeptide that is normally compartmentalized in the nucleus. For example, the
GFPZm-RADS1 fusion found in plasmidPHP8744 containsRADII, a protein required for mitotic and meiotic recombination and double-stranded break repair, fused to GFP. The gene encoding RADS1 was cloned from yeast.

Haaf et al., Proc. Nat Acad. Sci. 92:2298-2302 (1995).

Degenerate primers based on the nucleotide sequence of the RAD51 gene cloned from yeast were used to identify and isolate clones containing the RAD51 gene from maize. The carboxy terminus of GFPm was fused to the amino terminus of ZmRADS1a. Maize cells were transformed with the GFPm-ZmRADS1 fusion and fluorescence was localized in the nucleus. Plant regeneration and assessment of toxicity for this experiment has not yet been done.

Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those of ordinary skill in the art that various modifications may be made to the disclosed embodiments and that such modifications are intended to be within the scope of the present invention, which is defined by the following claims.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those in the art to which the invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference in its entirety.

Claims of WO9741223

What Is Claimed Is:

1. An isolated DNA molecule comprising a nucleotide sequence encoding a green fluorescent protein operably linked to an inducible promoter.

2. The isolated DNA sequence according to claim 1, wherein the inducible promoter is selected from the group consisting of the estrogen-inducible promoter, the estradiol-inducible promoter, the ACE1 promoter, the IN2 promoter and the tetracycline repressor promoter.

3. An isolated DNA molecule according to claim 1, wherein said nucleotide sequence encoding a green fluorescent protein is operably linked to a nucleotide sequence encoding a signal sequence for subcellular localization which directs a protein to a subcellular compartment.

4. An isolated DNA molecule comprising a nucleotide sequence selected from the group consisting of:
(a) SEQ ID NO: 1;
(b) a nucleotide sequence that has

substantial sequence similarity with SEQ
ID NO: 1; and
(c) a functional fragment of (a) or (b),
wherein said DNA molecule encodes a
green fluorescent protein.

5. An isolated DNA molecule according to claim 4, wherein said nucleotide sequence encoding a green fluorescent protein is operably linked to a nucleotide sequence encoding a signal sequence for subcellular localization which directs a protein to a subcellular compartment.

6. An isolated DNA molecule according to claim 5, further comprising a promoter operably linked to said nucleotide sequence encoding a signal sequence and a green fluorescent protein.

7. An expression vector comprising said DNA molecule of claim 1.

8. An expression vector comprising said DNA molecule of claim 4.

9. The expression vector according to claim 8, wherein said nucleotide sequence encoding a green fluorescent protein is operably linked to a nucleotide sequence encoding a signal sequence which directs a protein to a subcellular compartment.

10. The expression vector according to claim 9, further comprising a first promoter operably linked to said nucleotide sequence encoding a signal sequence and a green fluorescent protein.

11. The expression vector according to claim 10, further comprising a foreign gene, wherein said foreign gene is operably linked to a second promoter.

12. A method of using said expression vector of claim 7 to produce a transformed plant, comprising the steps of introducing said DNA molecule into regenerable plant cells, inducing expression of the gene encoding the green fluorescent protein, and selecting cells containing said green fluorescent protein for regeneration.

13. A method of using said expression vector of claim 8 to produce a transformed plant, comprising the steps of introducing said expression vector into regenerable plant cells and selecting cells containing said green fluorescent protein for regeneration.

14. The method of claim 12, wherein said regenerable plant cells are selected from the group consisting of Zea, Brassica and Helianthus cells.

15. The method of claim 13, wherein said regenerable plant cells are selected from the group consisting of Zea, Brassica and Helianthus cells.

16. A transgenic plant expressing the isolated
DNA molecule of claim 1.

17. A transgenic plant comprising the vector of claim 4.

18. A method for producing a transgenic plant, comprising the steps:
(a) constructing an expression vector
comprising (i) a first promoter which is
an inducible promoter, operably linked
to a nucleotide sequence encoding a
green fluorescent protein, and (ii) a
second promoter operably linked to a
foreign gene;
(b) introducing said expression vector into
regenerable plant cells;
(c) inducing expression of the gene encoding
the green fluorescent protein and
selecting transformed plant cells
containing said protein; and
(d) regenerating transformed plants from
said selected transformed plant cells.

19. The method of claim 18, where said inducible promoter is selected from the group consisting of the estrogen-inducible promoter, the estradiol-inducible promoter, the ACE1 promoter, the IN2 promoter and the tetracycline repressor promoter.

20. A method for producing a transgenic plant, comprising the steps:
(a) constructing an expression vector
comprising (i) a first promoter operably
linked to a nucleotide sequence encoding
a sequence for subcellular localization
which directs a protein to a subcellular
compartment and nucleotide sequence
encoding a green fluorescent protein,
and (ii) a second promoter operably
linked to a foreign gene;
(b) introducing said expression vector into
regenerable plant cells;
(c) selecting transformed plant cells
containing said green fluorescent
protein; and
(d) regenerating transformed plants from
said selected transformed plant cells.

21. The method of claim 20, wherein the sequence for subcellular localization is selected from the group which through targeting, signaling and/or retention localize the encoded protein to mitochondria, chloroplasts, peroxisomes, vacuole, endoplasmic reticulum, cell wall or for secretion generally into the apoplast.

22. The method according to claim 18, wherein said nucleotide sequence encoding a green fluorescent protein is selected from the group consisting of:
(a) SEQ ID NO: 1;
(b) a nucleotide sequence that has
substantial sequence similarity with SEQ
ID NO: 1; and
(c) a functional fragment of (a) or (b).

23. A method for producing a transgenic plant, comprising the steps:
(a) constructing an expression vector
comprising (i) a first promoter operably
linked to a nucleotide sequence encoding
a screenable marker flanked on the 5
prime and 3-prime ends with a
recombinase-specific target sequence,
and (ii) a second promoter operably
linked to a foreign gene;
(b) introducing said expression vector into
regenerable plant cells;
(c) selecting transformed plant cells
containing said screenable marker;
(d) transforming the plant cells with a
second expression vector containing a
gene encoding a site-specific
recombinase;
(e) selecting plant cells that no longer
express the screenable marker;
(f) regenerating transformed plants from
said selected transformed plant cells;
and
(g) isolating said foreign protein.

24. The method according to claim 23, wherein said site-specific recombinase is selected from the group consisting of the FLP/FRT, Ac/DS and cre/lox systems.

25. The method according to claim 23, wherein said screenable marker is the green fluorescent protein.

26. An isolated DNA molecule comprising a nucleotide sequence encoding the green fluorescent protein fused in frame to superoxide dismutase such that cells transformed with said DNA molecule contain a fusion protein that (i) produces green fluorescence in the presence of W to blue light and (ii) displays superoxide dismutase activity.
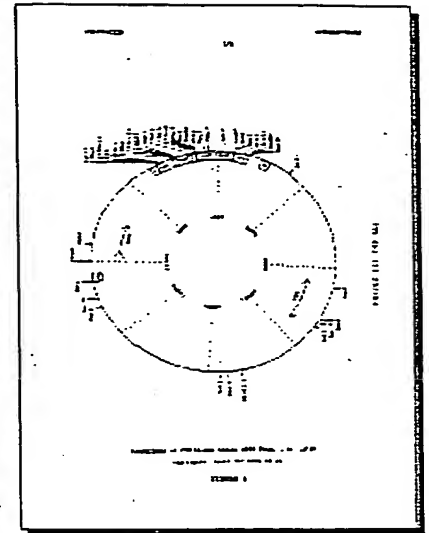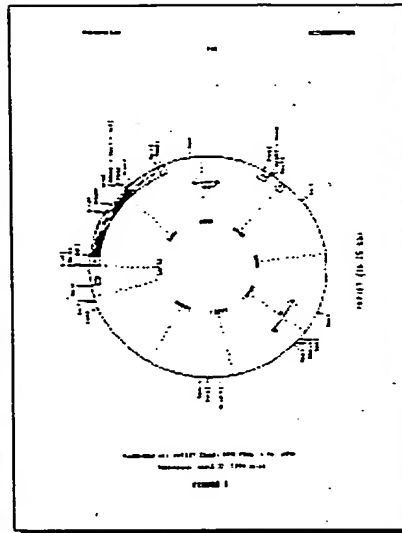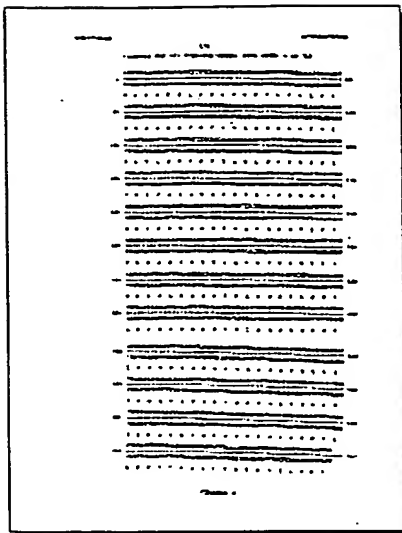
27. A method for producing a transgenic plant comprising:
(a) constructing an expression vector comprising (i) a first promoter operably linked to a nucleotide sequence encoding a green fluorescent protein, and (ii) a second promotercperably linked to a gene encoding an enzyme that is an oxygen scavenger, and (iii) a third promoter operably linked to a foreign gene;
(b) introducing said expression vector into regenerable plant cells;
(c) selecting transformed plant cells containing said green fluorescent. protein; and
(d) regenerating transformed plants from said selected transformed plant cells.

28. The method aecording to claim 27, wherein said oxygen scavenger enzyme is superoxide dismutase.

29. A method for producing a transgenic plant comprising:
(a) constructing an expression vector comprising (i) a first promoter operably linked to a nucleotide sequence encoding a fusion protein comprising the green fluorescent protein and an oxygen scavenger enzyme fused in frame, and (ii) a second promoter operably linked to a foreign gene;
(b) introducing said expression vector into regenerable plant cells;
(c) selecting transformed plant cells containing said green fluorescent protein and oxygen scavenger enzyme activity; and
(d) regenerating transformed plants from said selected transformed plant cells.

30. The method according to claim 29, wherein said oxygen scavenger enzyme is superoxide dismutase.

Drawing pages of WO9741228